MICROFLUIDIC SYSTEMS FOR HIGH-THROUGHPUT BIOPHYSICAL CHARACTERIZATION OF SINGLE CELLS

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

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Biophysical (mechanical and electrical) properties of living cells have been proven to play important roles in the regulation of various biological activities at the molecular and cellular level, and can serve as promising label-free markers of cells’ physiological states. In the past two decades, a number of research tools have been developed for understanding the association between biophysical property changes of biological cells and human diseases; however, technical challenges of realizing high-throughput, robust and easy-to-perform measurements on single-cell biophysical properties have yet to be solved. This thesis focuses on the development, testing and modeling of microfluidic platforms for biophysical characterization of single cells.

The proposed microfluidic system for biophysical characterization of red blood cells (RBCs) achieved a speed of 100-150 cells/second and was capable of quantifying multiple
parameters as mechanical and electrical signatures of each RBC including transit time, impedance amplitude ratio, and impedance phase increase. In comparison with previously reported microfluidic devices for single RBC biophysical measurement, this system has a higher throughput, higher signal to noise ratio, and the capability of performing multi-parameter measurements. The microfluidic device consisting of two stages of microchannels was also developed for measuring mechanical opacity to mitigate the coupled effect of cell size/volume and deformability. The stiffness/deformability changes of lymphocytes in chronic lymphocytic leukemia (CLL) patients were, for the first time, studied using this system.

In order to extract the inherent electrical properties of cells, electrical and geometrical models are developed to interpret the impedance data and to determine the specific membrane capacitance and cytoplasm conductivity of individual cells. Results from testing 3,249 AML-2 cells and 3,398 HL-60 cells reveal different specific membrane capacitance and cytoplasm conductivity values between AML-2 (12.0±1.44 mF/m², 0.62±0.10 S/m) and HL-60 (14.5±1.75 mF/m², 0.76±0.12 S/m) cells. The results also demonstrate that the quantification of specific membrane capacitance and cytoplasm conductivity can enhance cell classification results since these parameters contain information additional to cell size.

The progressive deformability changes during blood banking/storage were studied using a microfluidic system. High-speed imaging (5,000 frames/sec) captures the dynamic deformation behavior of the cells, and together with automated image analysis, enables the characterization of over 1,000 RBCs within 3 minutes. Multiple parameters including deformation index (DI), time constant (shape recovery rate), and RBC circularity were quantified. Compared to previous studies on stored RBC deformability, our results include a
significantly higher number of cells (>1,000 cells/sample vs. a few to tens of cells/sample) and, for the first time, reveal deformation changes of stored RBCs when traveling through human-capillary-like microchannels.
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Chapter 1

1. Introduction

1.1 Background

The cell, the basic functional unit of living organisms, maintains and senses the physiological environment within the organism both chemically and physically [1-4]. The unique biochemical and biophysical properties enable a cell to fulfill its specific functions and adapt to its surrounding environment. Physiological changes within the cells are accompanied by chemical and physical modifications and reorganization. Thus, pathological cells can be identified biochemically and/or biophysically. Biochemical properties of pathological cells have been under intensive study, and many biochemical markers have been developed to identify target cells out of a heterogeneous population [5, 6]. However, although research on physical properties of cells has provided strong evidence about the capability of physical properties as potential markers for identifying cell types, most of biophysics research was limited to proof-of-concept demonstrations. The lack of clinical relevance is due to the very low measurement throughput and tedious operation procedures of conventional techniques for measuring the biophysical properties of cells [4, 7-9]. Compared to conventional
techniques, the advantages of small sample volume, integration capability, biocompatibility and fast response make microfluidic technologies attractive for studying cells. Recent decades have witnessed significant advances of microfluidic technologies for biochemical characterization of cells [10, 11]. Microfluidics is extending its way to the characterization of single-cell biophysical properties [12-14].

1.2 Mechanical Characterization Techniques

The association between cell deformability and human diseases has been of interest since the 1960s [15, 16]. The deformability of nucleated cells is determined by the membrane, the cytoskeletal network (actin filaments, intermediate filaments, and microtubules), and its interaction with the nucleus, while the deformability of red blood cells (RBCs) is determined by the membrane skeleton network and the interaction between the membrane skeleton and membrane integral proteins [4, 17, 18]. Physiological and pathological changes can alter the cytoskeleton composition, reorganize the network structure, and change the protein density. As a result, cell deformability can be used as an intrinsic marker for identifying pathological conditions [8, 19, 20]. For example, deformability is known to play a crucial role in the mobility of cancerous cells [4, 21]; and RBC deformability decrease has been proven relevant to several human diseases.

1.2.1 Structure-induced deformation (Constriction channel)

Constriction channels, which are marginally smaller than the diameters of tested cells, provide an efficient method to generate mechanical stimuli. Cells driven through a constriction channel are squeezed by the walls of the constriction channel. Multiple parameters, such as transit time, elongation and recovery time, in association with cell
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deformability can be quantified. Moreover, constriction channels can be easily fabricated with standard microfabrication techniques and are able to provide an environment to mimic in vivo capillaries. With the use of high-speed imaging or electrical impedance measurements, constriction channel devices are capable of achieving a higher throughput than most other deformability measurement approaches. Due to these merits, constriction channels have been used to measure the deformability of RBCs [22-29], leukocytes [30] and cancer cells [31, 32].

Due to the human capillary-like environment and the physiological relevance of RBC deformability, RBC is studied most in the majority of existing constriction channel-based devices. The first demonstration of microfluidic constriction channels for RBC deformability characterization was reported in 2003 [22]. In this study, constriction channels with various diameters were used to study the deformability changes between healthy RBCs and malaria parasite infected RBCs at different stages (early ring stage, early trophozoite, late trophozoite, and schizont). They found that the deformability of malaria-infected RBCs decreases as the parasite progresses from the early ring stage to a schizont, while healthy RBCs are exceptionally deformable and even able to travel through the constriction channels blocked by infected RBCs. In addition to RBC deformability, white blood cell (WBC) deformability was also studied using microfluidic constriction channels. Rosenbluth et al. demonstrated the clinical relevance of their microfluidic constriction channel device in sepsis and leukostasis [30]. The reported device consists of a network of 64 constriction channels. High-speed imaging was used for measuring cell transit time. They used patient samples to show that cell transit time increased for diseased samples compared to control samples (Figure 1.1 (a)). In another study, breast cancer cell lines (MCF-7 and MCF-10A) were flown through a
constriction channel to distinguish nonmalignant and malignant cells [31]. According to the reported results, transit velocity is not significantly affected by cell types, and the transit time difference is mainly determined by the entry time. Different cell types can be distinguished on the basis of scatter plots of cell volume and entry time.

Besides imaging, microfluidic constriction channel can also be used together with other measurement techniques to achieve multiple parameter measurements for cell type classification. We recently developed a microfluidic device (Figure 1.1 (b)) which combines a constriction channel and impedance measurements [23]. Detection involves only electrical signals; hence, it enables a throughput higher than 100 cells/sec. The multiple parameters quantified as mechanical and electrical signatures include transit time, impedance amplitude ratio, and impedance phase increase. Histograms compiled from 84,073 adult RBCs and 82,253 neonatal RBCs reveal different biophysical properties across samples and between the adult and neonatal RBC populations. Bow et al. demonstrated a deformability-based RBC testing device combining constriction channels and fluorescence measurement. They showed a population-based correlation between biochemical properties, such as cell surface markers, and mechanical deformability [24]. They also showed experimentally that entrance geometry of the constriction channel has a significant impact on RBC transit time, and developed a dissipative particle dynamics model to interpret the parasite effect on RBC deformability. An optical stretcher was also introduced to enhance the sensitivity and reliability of a constriction channel based microfluidic device [25]. Scatter plots compiled from transit time, elongation, and recovery time measured by this device proved effective for the discrimination of RBCs from normal donors and leukemia patients.
Other applications of microfluidic constriction channels include the usage of wedge-shaped constriction channels to measure the surface area and volume of a large population of RBCs [26-28], and a microfluidic manometer to measure the pressure drop due to the presence of a cell in the constriction region, which correlates with the stiffness of the cell, by observing the displacement of downstream fluid-fluid interface [29]. Despite the advantages of the constriction channel technique, cell volume and adhesion between cell membrane and channel walls are coupled with cell deformability. Consequently, longer transit time does not necessarily mean lower deformability since larger and stickier cells can also lead to longer transit time. Efforts have been made to take cell volume/size effect into account. For example, Adamo et al. reported a microfluidic device for probing both cell volume and transit time. Comparisons of cell transit time were made among cells with similar volume [33]. They demonstrated that Hela cells in the control group have longer transit time than Hela cells treated with latrunculin A and cytochalasin B. The size of the constriction channel can also be adjusted on demand according to targeted cell size (MCF-7 and MCF-10A) [34]. However, there exists no technique that is capable of characterizing the adhesion (or friction) between cell membrane and channel walls. In addition, since the diameter of the constriction channel is smaller than the diameter of targeted cells, the channel is susceptible to clogging. Using an array of constriction channels appears to be a potential solution to the clogging issue.

1.2.2 Fluid-induced deformation

RBCs are highly deformable and can easily deform under fluid shear stress inside blood vessels. Having micrometer dimensions, which are comparable with in vivo capillaries, microfluidic channels provide an ideal tool for investigating RBC deformability. Compared to constriction channel, the microfluidic channels used to generate shear stress are larger than
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RBCs. Thus, RBCs are deformed by fluid shear stress instead of channel’s confinement structures. The deformation index (DI) quantified via high-speed imaging is often used to indicate RBC deformability. Forsyth et al. studied the deformability and dynamic behavior of chemically “stiffened” RBCs using a simple straight microfluidic channel, revealing three different types of motion due to the increased shear rate in the microfluidic channel: stretching, tumbling, and recoiling [35]. Besides straight channels, a hyperbolic converging microchannel was also developed for assessing RBC deformability by measuring extensional flow-induced deformation [36]. The results confirmed that extensional flow is more efficient in causing RBC deformation. Shear stress generated in microfluidic channels was also used to measure the dynamics of shear-induced adenosine triphosphate (ATP) release from RBCs. In [37], RBCs were driven through a microfluidic channel with a cross-sectional area of 20µm×20µm, while the amount of ATP released was measured by counting the photons emitted from standard luciferase-ATP bioluminescent reaction. Cell deformation and dynamics were quantified simultaneously using high-speed imaging.

Although shear stress proves effective for investigating RBC deformability, the low magnitude of shear stress is typically not able to deform other types of cells. Gossett et al. recently reported a hydrodynamic stretching microfluidic device for identifying malignant cells in human pleural fluid sample with a measurement speed of ~2,000 cells/sec (see Figure 1.1 (c)) [19]. Cells are focused towards a narrow streamline near the center of the microfluidic channel and delivered to a junction of two orthogonal channels at a high flow rate, where the cells undergo mechanical stretching. In the meanwhile, cell deformations are captured using a high-speed camera and images are then analyzed to extract cell volume and deformation index (DI). This approach achieved both high testing speed and larger...
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deformation of tested cells. The authors predicted disease states in patients with cancer and immune activation with a sensitivity of 91% and a specificity of 86%.

In fluid-induced deformation based microfluidic devices, channel dimensions are larger than cell diameters. DI is used as the deformability indicator and is not affected by the adhesion between cell membrane and channel walls (vs. constriction channel). However, since cells of different sizes may experience different forces due to the non-uniformity in the shear stress and hydrodynamic pressure within the microchannel, DI is still associated with cell volume. More specifically, shear stress is highest near channel walls and is almost zero in the center of the channel. As a result, RBCs with larger volumes experience higher shear stress on the edges causing larger deformation compared to cells with smaller volumes. Within the hydrodynamic stretching microfluidic device, the highest compressive pressure appears at the center of the junction where the cells are stretched, which causes the cells with different volumes or shapes to be exposed under different pressure environment. To address this issue, cell volume is measured as an independent parameter and used as a reference for cell type classification [19].

A limitation of fluid-induced deformation based microfluidic devices is the use of high-speed imaging (tens of kHz) for measuring DI. High-speed cameras are often costly and must be used on a microscope, making the overall microfluidic system bulky. Furthermore, since real-time image data transfer from a high-speed camera to the computer hard drive cannot be achieved, images are stored in the camera’s on-board memory for later off-line transfer. Since state-of-the-art high-speed cameras typically have only a few GB on-board memory, after recording for a few seconds, the experiment must be stopped for image data transfer. Processing massive amounts of image data also costs tremendous computational effort and
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time. Advances in high-speed cameras and hardware-based image processing techniques, such as FPGA-based techniques, will possibly be able to solve the data transfer problem. Additionally, opto-microfluidics, microfluidic devices integrated with optical comments, also seems promising to tackle this problem. Integrating on-chip lenses and CMOS chips can achieve real-time performance and in the meanwhile, eliminate the need of the use of bulky microscopes [38].

1.2.3 Electroporation-induced deformation

Electroporation is a technique used for introducing foreign molecules, such as DNA and proteins, into cells. The concept of electroporation capitalizes on the relatively weak nature of the phospholipid bilayer’s hydrophobic/hydrophilic interactions and its ability to spontaneously reassemble after disturbance [39]. Thus, a voltage shock can disrupt areas of the membrane temporarily, allowing polar molecules to pass. The membrane then can reseal and leave the cell intact [39-41]. Swelling or expansion in cell size accompanies the cell’s electrical property changes [42], which is caused by the influx of molecules through the open pores in the cell membrane (vs. dielectric force in DEP-induced deformation). Recently, a few studies correlated electroporation-induced cell deformation and lysis to cell deformability changes. Bao et al. [43] developed microfluidic electroporative flow cytometry to study the deformability of single cells. A constant DC voltage was established across the microchannel, which concentrated the electric field to yield repeatable cell exposure to uniform electrical fields. When cells were flown through the microchannel, the swelling of cells was recorded by imaging and quantified as a deformability indicator. Deformability changes of breast cancer cell lines [43] and the expansion of the nuclei of circulating tumor cells (CTCs) were tested using the system [44]. The capability to detect
RBCs with deficiencies of cytoskeletal protein network was also demonstrated (see Figure 1.1 (d)) [45]. The achieved throughput was about 5 cells/sec.

It is notable that electroporation efficiency is also dependent on cell volume. Under the same uniform electrical field, the effective voltage across the cell membrane is a function of cell diameter [46]. Hence, cells with larger diameters are exposed to higher voltages and are more easily porated. In addition, the plasma membrane of different cell types (e.g., metastatic tumor cells vs. non-metastatic tumor cells) can possess different poration properties. In other words, some cell types may be more susceptible to electrical fields than others. Thus, based on electroporation-induced expansion or swelling alone, it can be inaccurate to conclude that cell deformation is caused by cell deformability rather than the membrane’s poration susceptibility to electrical voltages.

1.2.4 Optical stretcher

Optical trapping was discovered in 1970 [47] when radiation pressure from laser light was found to be able to accelerate and trap micron-sized dielectric particles. Light interacts with a particle by imparting some of its momentum onto it, thus exerting a force on the particle. If the particle is not centered on the optical axis of the beam, a restoring force is exerted on the particle to keep it on the optical axis. Optical stretchers utilize two slightly divergent Gaussian beams to trap an object in the middle. This setup was used to deform and measure the stress profile of erythrocytes [48], which led to the first optical stretcher device reported in 2001 [49] as a novel method to stretch biological cells and probe their viscoelastic properties.
Figure 1.1 (a) A network of bifurcating microfluidic channels for blood cell deformability measurement. The transit time of the individual cells are measured using a high-speed camera. Reproduced with permission from [30]. (b) A microfluidic system for electrical and mechanical characterization of RBCs at a speed of 100-150 cells/sec. The transit time is obtained from electrical impedance data captured during RBCs flowing through the constriction channel. Reproduced with permission from [23]. (c) Hydrodynamic stretching microfluidic device. Cells are focused to the center lines of the channels by inertial force and stretched by fluid pressure. Cell deformation is measured by analyzing images recorded by a high-speed camera. Reproduced with permission from [19]. (d) Electroporation-induced RBC lysis. Time-lapse images of RBCs are recorded by a high-speed camera as the cells flow through the channel while a constant DC voltage is applied. Cell lysis time is correlated with RBC deformability. Reproduced with permission from [45].
Figure 1.2 (a) Optical stretcher. Cells travel along a flow channel integrated with two opposing laser beams. Cells are trapped and deformed optically, and cell deformation images are recorded. Reproduced with permission from [50]. (b) Single-cell microchamber array for electro-deformation. An array of microchambers integrated with DEP electrodes is used to trap individual RBCs and apply DEP force. Reproduced with permission from [51]. (c) Microfluidic pipette aspiration of cells using funnel channel chain. Reproduced with permission from [52].
A typical optical stretcher system consists of a microchannel for loading cells into the testing region and two laser fibers located on the sides of the passageway (see Figure 1.2 (a)) [50]. Cells flowing through the microfluidic channels are serially trapped and deformed with the two counter propagating divergent laser beams [53, 54]. For trapping and stretching cells in the optical stretcher, the alignment of the fibers is crucial. In order to improve alignment, a platform capable of adjusting fiber positions was developed [55] using pneumatically driven manipulators. Femtosecond laser technique has been utilized for the fabrication of the optical stretcher microfluidic devices [56, 57]. By direct writing of both optical waveguides and microfluidic channels, the alignment problem can be mitigated significantly. The deformability of human cancer cell lines [53, 54], red blood cells [55] and patient oral squamous cell [58] was characterized using optical stretchers.

1.2.5 DEP-induced deformation

When polarized in an electric field, biological cells will experience dielectric force, which is well known as DEP Force [59, 60]. Electro-deformation devices utilize DEP force for trapping and generating mechanical stimuli to quantify the deformability of individual cells [61, 62]. The Young’s modulus of tested cells can be extracted with either analytical models or numerical simulation. For improving the throughput of electro-deformation devices, a single-cell microchamber array device [51] was proposed. The device is able to trap individual RBCs in a large array of micro-wells integrated with DEP electrodes. The ITO electrodes also allow the correlation of RBC deformation with cell surface and cytosolic characteristics (Figure 1.2 (b)). Even a single-cell microchamber array was adopted, the overall throughput is still quite limited due to the time-consuming cell trapping procedure. On the other hand, the complex physical phenomena involved in electro-deformation and
unknown cell electrical properties pose difficulties in extracting forces experienced by an electro-deformed cell [59, 61, 62]. Since DEP force is determined by the dielectrical properties of cells, DEP-based techniques can also be used to electrically characterize cells, which is discussed in the Electrorotation section under electrical characterization.

1.2.6 Aspiration-induced deformation

Pipette aspiration is a conventional technique for studying mechanical properties of single cells [63]. The mathematical models in conventional pipette aspiration have been adopted in microfluidic characterization of cells. Figure 1.2 (c) shows a microfluidic pipette aspiration device [52]. Single cells are infused into a microfluidic channel and deformed through a series of funnel-shaped constrictions. Malaria infected RBCs were tested using both membrane cortical tension and threshold pressure as readouts. We developed a microfluidic device for single-cell electrical and mechanical characterization using impedance spectroscopy and micropipette aspiration [64]. Cellular deformation was recorded as a function of increasing pressure while cellular impedance was measured via two Ag/AgCl electrodes inserted into the culture medium. With pipette aspiration and equivalent circuit models, both mechanical properties and dielectric properties of single cells were quantified. In addition to throughput limitation, the rectangle-like cross-section in microfluidic pipette aspiration devices can cause concerns about the validity of applying conventional pipette aspiration models.

1.2.7 Compression-induced deformation

Compressive forces can be applied directly to cells through a mechanism similar to bulging elastomeric valves [65]. In general, such devices consist of multiple layers and a thin
elastomeric membrane separating the flow and control channels. The cells within the flow channels are compressed when a pneumatic pressure is applied to deflect the thin membrane. Microfluidic devices based on the compression have been used to monitor cell viability and induce mechanical cell lysis of adherent breast cancer cells (MCF-7) [66]. A more recent study by Kim et al. has shown that under excessive deformation of the deflecting membrane, small morphological changes known as “bulges” occur on the cellular membrane as a result of local detachment of the cytoskeleton lipid bilayer. In addition, differences in the uniformity of bulge distribution on the cell periphery can be used as a physical indicator to distinguish different cells [67]. A similar technology can also be used for viscoelastic characterization of cells of a small population in suspension. Du et al. estimated global viscoelastic time constants of HL-60 cell line and 3T3 fibroblast by observing the recovery time upon the removal of the compression force [68]. This design can also be extended to the mechanical characterization of other specimens such as biofilms [69]. Alternatively, hydrostatic pressure can apply a uniform compressive force without physical contact [70].
Figure 1.3 (a) Schematic illustration of PGEs-based DC impedance cell counter. Ionic flows between the PGEs under low DC bias are interrupted when a cell passes through, causing a DC impedance change. The sheath flow is used for focusing cells and preventing cell adhesion to chambers and channels. Reproduced with permission from [71]. (b) Electrorotation. A cell is placed in the center of four micro-electrodes connected in phase quadrature (90° difference) and rotated by the DEP forces generated by the rotating electrical field. Rotation spectra (rotation rate vs. frequency) are used to extract the electrical properties of the cells. Reproduced with permission from [72]. (c) Schematic of the μEIS system showing four impedance analysis sites positioned along the two cell flow channels. The close-up view illustrates a single cell trapped inside a cell trap. The trapped cell forms a tight seal with the two integrated electrodes for impedance measurement. Reproduced with permission from [73].
1.3 Electrical Characterization Techniques

Besides mechanical deformability, electrical properties of cells are also important physical properties, serving as the basis of counting, trapping, focusing, separating, and the characterization of single cells [74, 75]. Early work on cell electrical measurements dates back to the 1910s [76-78], when approximate hemoglobin conductivity inside RBCs was reported. The single shell model proposed by Pauly and Schwan in the 1950s laid the foundation for interpreting electrical properties of cells, where the cell is modeled as a spherical cytoplasm surrounded by a thin dielectric membrane [79, 80]. Generally, plasma membrane’s electrical properties are affected by the membrane morphology, lipid bilayer composition and thickness, and embedded proteins [81-83]. Cytoplasm’s electrical properties are influenced by the intracellular structures and physiological conditions (e.g., nucleus-to-cytoplasm ratio and ion concentrations inside the cell) [84-86]. Early techniques are only capable of measuring average electrical properties of a cell population. The most successful example of electrical measurements on single cells is the Coulter counter. However, direct current (DC) measurements are limited to counting and sizing single cells. The patch-clamp technique developed in 1976 is capable of electrical characterization of single cells [87]. However, patch-clamp typically takes tens of minutes for testing one cell. Because of the capability of manipulating micro-scaled objects and integrating micro electrodes on chip, microfluidic techniques have gained momentum in single cell electrical characterization in recent years.
1.3.1 Microfluidic Coulter counter

The Coulter counter monitors the DC resistance of a small orifice as micro particles passing through. Since the cell membrane acts as an insulating layer at DC, the presence of the cell alters the resistance of the orifice by replacing the conductive liquid. Well established models are available for correlating DC resistance changes to cell volumes. Several designs have recently been proposed to improve microfluidic Coulter counter’s performance. For instance, two-dimensional sheath flow focusing was demonstrated to overcome the clogging issue [88]; and throughput of microfluidic Coulter counters was improved using multiple-orifice designs [89].

One challenge for microfluidic Coulter counters lies in the selection of the electrode material. Ag/AgCl non-polarizable electrodes, which work well in the conventional Coulter counter, are an ideal choice. Nonetheless, integrating Ag/AgCl electrodes into microfluidic channels is complex, and Ag/AgCl electrodes have limited lifetime [90, 91]. Although other metal electrodes can be more readily integrated into microfluidic channels, the electrical double layers formed between the interface of electrodes and liquid, which are mainly capacitive, pose difficulties in applying DC signals. Methods for minimizing the electrical double layer effect include the modification of the electrode surface roughness in order to increase the surface area [92] and the utilization of polyelectrolytic salt bridges (PSBES) [93] or polyelectrolyte gel electrodes (PGEs) [94]. Most recently, a DC impedance-based microcytometer device integrating PGEs was reported for CTC cell detection. Sheath flow is used for focusing cells and preventing cell adhesion to chambers and channels (see Figure 1.3 (a)). CTCs were successfully detected in blood samples from breast cancer patients [71]. Besides the electrode design that demands special design consideration, the model used in the
conventional Coulter counter to calculate particle volumes is not directly applicable to microfluidic Coulter counter devices, due to the different electrode configuration and channel geometry [95, 96].

### 1.3.2 Electrorotation

When a cell/particle is placed in a non-uniform electrical field, a force is exerted on the induced dipole and can cause the cell to either move (DEP) or rotate (ROT) [59, 72]. Maxwell’s mixture theory is commonly used to interpret DEP and ROT data, for associating the complex permittivity of the suspension to the complex permittivity of the particle/cell [59, 72]. The DEP force and ROT torque are proportional to the real and imaginary parts of the Clausius–Mossotti factor, respectively [9]. Although DEP and ROT are closely related, ROT is more suitable for single cell electrical characterization since in ROT, the cells are only rotated at a certain position in the electric field. Thus, the amplitude of the electric field remains unchanged, which is suitable for fitting the rotation spectra to determine intrinsic electrical properties of single cells (e.g., specific membrane capacitance, cytoplasm conductivity and cytoplasm permittivity). Differently, forces in DEP are determined by the electric field gradient. A constant electric field gradient is technically challenging to achieve. Furthermore, the rotation rate is only determined by the rotation force (constant as a cell rotates) and the viscosity of the suspension medium in ROT, which can be readily measured, whereas the DEP force (varying as a cell moves) is difficult to quantify. DEP was demonstrated to electrically characterize cells by curve fitting of the Clausius–Mossotti factor spectra or cell count spectra. However, since the spectra are not from the measurements of the same cells, only average electrical properties of a cell population can be
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obtained [97-99]. More details on DEP and ROT models can be found in other reviews [9, 100].

The most popular ROT setup uses four electrodes connected to sine waves in phase quadrature [81, 101]. Cells are placed in the center of the four electrodes (e.g., using laser tweezers). Rotation spectra are obtained by measuring the rotation speed at each frequency over a frequency range of 1 kHz to around 100 MHz. By fitting the rotation spectra, specific membrane capacitance, cytoplasm conductivity and cytoplasm permittivity values are determined [82, 102, 103]. Figure 1.3 (b) illustrates the working mechanism of a microfluidic electro-rotation device [72]. The ROT technique has been used to test a number of cell types, such as white blood cells and human cancer cells [81, 82, 102-104]. Electrorotation is a slow technique. It takes approximately 30 minutes to test a single cell [101, 105]. It is also difficult to achieve efficient rotation in a high conductivity physiological buffer. Hence, electrical properties of the tested cell may have already been altered when immersed in the low conductivity sucrose buffer. Nevertheless, electrorotation is the only method capable of extracting inherent electrical properties of cells, such as membrane permittivity and cytoplasm conductivity. The methodology and electrical models can be instrumental for the development of new techniques of higher throughput.

1.3.3 Micro electrical impedance spectroscopy (µ-EIS)

Micro electrical impedance spectroscopy (µ-EIS) is a technique wherein a frequency-dependent excitation signal is applied across a trapped cell to measure the corresponding current response. Various cell trapping mechanisms incorporated with microfabricated electrodes have been proposed, such as hydrodynamic traps [106, 107], negative pressure traps [73, 108], and DEP traps [109].
Jang et al. developed a microfluidic device which utilizes micro pillars within a microfluidic channel to physically capture and measure the impedance of a single human cervical epithelioid carcinoma (HeLa) cell using EIS [106]. Hydraulic trapping devices were also demonstrated where impedance measurements were accomplished by recording current from two electrode pairs, one empty (reference) and one containing a cell. The effect of surfactant and bacterial pore-forming toxins on HeLa cells was monitored continuously [107]. For minimizing current leakage, caused by the current undesirably obviating the targeted cell through the low resistivity medium, Cho et al. developed an array of planar micro holes for positioning cells and forming direct contact between cells and electrodes (see Figure 1.3 (c)) [73]. Li et al. used a vertical sub-micrometer opening with embedded recording electrodes. The device was capable of reducing the serial resistance while keeping the seal resistance for high sensitivity measurements [110]. Since single cells can be cultured right on the vertical holes, the capability of monitoring the dynamic changes of single cell electrical properties over a period of time is a merit of the vertical hole-based technique [111, 112].

There are two major limitations of the µ-EIS technique. Firstly, since the trapping and releasing process is time consuming, and the measurement of impedance spectra also takes time, the throughput of µ-EIS is low. Only tens of cells were tested in reported studies. Secondly, although the capacitance and resistance of tested cells can be determined by interpreting impedance spectra with electrical models, these parameters are strongly affected by electrode size, the cell trapping mechanism, cell volume and the interaction with other cells (vs. size-independent parameters such as specific membrane capacitance and cytoplasm conductivity).
1.3.4 Impedance flow cytometry (IFC)

Although the Coulter counter has become a widely used technique in clinical instruments (e.g., hematology analyzers), the conventional Coulter counter is only capable of classifying cell types that have distinct volume differences. Some of the most recent commercial hematology analyzers adopted both DC and RF measurements. The ratio of the RF signal to the DC signal is defined as opacity, which is used as a volume independent electrical signature of the tested cells. Along with the DC signal (volume signature), WBC differentials count can be achieved. This technique was recently demonstrated on microfluidic devices. In addition to WBC count, microfluidic flow cytometry has also been used for analyzing a variety of particles [113-117].

An impedance flow cytometry design using coplanar electrodes was demonstrated by Gawad et al. [113]. As shown in Figure 1.4 (a), three microfabricated electrodes were integrated on the bottom of the microfluidic channel. When micro particles were flowing through the detection area, impedance change was recorded differentially. The non-uniform electrical field distribution in the channel resulted in position variations of tested cells may undesirably affect impedance measurement. To overcome this issue, parallel facing electrodes were developed (see Figure 1.4 (b)) [114]. When a cell is present in between one pair of electrodes, the other pair is used as reference, allowing the differential measurement of electrical signals. The device was demonstrated capable of detecting electrical property differences between normal RBCs and glutaraldehyde-fixed RBCs.

Homes et al. incorporated a fluorescence measurement unit on their microfluidic flow cytometry device [116]. This device design enabled direct correlation of impedance signals from individual cells with their biochemical phenotypes. 3-part WBC differential count of
human blood was well achieved. The same group more recently developed a microfluidic impedance flow cytometry device integrated with RBC lysis and a hemoglobin concentration measurement unit [118, 119]. Besides counting blood cells, microfluidic impedance flow cytometry has also found applications in other areas, such as the measurement of cell viability and physiological differences of cells [117]. More details on impedance flow cytometry can be found elsewhere [100, 117]. In addition to coplanar and parallel facing electrode designs, Mernier et al. demonstrated “liquid electrodes” to discriminate living and dead yeast cells (see Figure 1.4 (c)). The larger electrodes recessed in lateral channels allow measurements at low frequencies (down to 1 kHz), and the same “liquid electrodes” can also be used for DEP focus of the particles [120]. Recently, the concept of label-free electrophysiological cytometry was reported. This technique utilizes the nature of electrically-excitable cells (upon activation by sufficient trans-membrane electric fields, different cell types generate different extracellular field potential signals) to distinguish different cell types. As shown in Figure 1.4 (d), the device is integrated with electrodes for both electrical stimulation and recording of extracellular field potential signals from suspended cells in flow [121]. Undifferentiated human induced pluripotent stem cells (iPSC) and iPSC-derived cardiomyocyte (iPSC-CM) cells were distinguished.
Figure 1.4 (a) Impedance flow cytometry with coplanar electrodes. Side view shows a particle passing over three electrodes (A, C, and B). Impedance signal is measured differentially ($Z_{AC} - Z_{BC}$). Reproduced with permission from [113]. (b) Impedance flow cytometry with parallel facing electrodes. Side view shows a cell passing between the measurement and reference electrodes. Reproduced with permission from [114]. (c) Impedance flow cytometry combining impedance measurements and dielectrophoretic focusing. Reproduced with permission from [120]. (d) Label-free electrophysiological cytometry. Electrodes are integrated for both electrical stimulation of cells and recording of their extracellular
1.4 Research Objectives

The overarching goal of the thesis is to develop novel microfluidic systems for high-throughput biophysical properties characterization of single biological cells. Specific objectives include:

- To design, prototype, and characterize microfluidic systems for high-throughput biophysical (mechanical and electrical) characterization of single cells.
- To develop microfluidic systems for high-throughput measurement of specific membrane capacitance and cytoplasm conductivity of single cells.
- To study deformability changes of red blood cells during blood storage in human-capillary-like environment.

1.5 Dissertation Outline

An overview of the ensuing chapters is as follows: Chapter 2 describes the development of a microfluidic system for simultaneous biophysical properties measurement of red blood cells. Chapter 3 presents a technique for electrical measurement of red blood cell deformability on a microfluidic device. Chapter 4 describes the microfluidic system for high-throughput measurement of specific membrane capacitance and cytoplasm conductivity of single cells. In Chapter 5, the characterization of red blood cell deformability change during blood storage is presented. Chapter 6, the stiffness/deformability changes of lymphocytes in chronic lymphocytic leukemia (CLL) patients are studied. The thesis is concluded in Chapter 7, with a summary and contributions of this research and suggested future research directions.
Chapter 2

2. High-Throughput Biophysical Measurement of Human Red Blood Cells

2.1 Introduction

Because of the physiological and pathological importance of RBCs, their biophysical properties (mechanical and electrical properties) have been under intensive study over the past decades [15, 16, 122]. The mechanical property of RBCs is essentially determined by the membrane skeleton, and the interaction between the membrane skeleton and membrane integral proteins [123] [17]. RBCs’ exceptional capacity to deform is of crucial importance to both macro and microcirculation. The high deformability of normal RBCs enables them to pass capillaries that are smaller than the diameter of RBCs. A range of diseases have been described in association with impaired RBC deformability, such as sepsis [124] [125], malaria [24] [22], sickle cell anemia (hemoglobin disorder) [126] [127], and myocardial ischaemia and microvascular dysfunction [128]. In the meanwhile, the electrical properties of RBCs have also been correlated to pathological conditions [129-131]. For example, the ion
channel conductance of malaria parasite infected RBCs is lower than the uninfected RBCs due to the blockage of ion channels by the parasites [132].

A number of technologies have been used for measuring single RBC’s biophysical properties [13, 133], for instance, micropipette aspiration [63], atomic force microscopy [134, 135], and optical tweezers [136, 137] for mechanical measurement and patch-clamping for electrical measurement [14] [16]. However, these technologies are difficult to use and have a low testing speed. The heterogeneity of RBCs within a sample demands a higher testing throughput in order to obtain statistically significant data, and thus, determine RBCs’ genuine mechanical and electrical properties.

This chapter presents a microfluidic system for simultaneous mechanical and electrical characterization of single RBCs. Detection involves only electrical signals and has a throughput of 100-150 cells per second. Since adult and fetal/neonatal RBCs are known to be different in size and hemoglobin contents, we applied our system to test RBCs in adult blood (~16,000 cells/sample, 5 samples) and neonatal RBCs (~16,000 cells/ sample, 5 samples) and revealed their differences in mechanical and electrical properties. We also explored the utility of biophysical (mechanical and electrical) data for distinguishing neonatal RBCs from adult RBCs.
Figure 2.1 (a) Schematic of the microfluidic system for electrical and mechanical characterization of RBCs. Two Ag/AgCl non-polarizable electrodes are connected to a function generator (100 kHz @1.0 V_{pp}) and a lock-in amplifier. The two electrodes are inserted into the inlet and outlet ports of the device. As RBCs are aspirated through the constriction channel, electrical current change is sensed and amplified. “-P” denotes the negative pressure used to aspirate RBCs through the constriction channel. (b) Measurements are made when an RBC passes through the constriction channel. (c) Equivalent circuit model of the system.
2.2 System overview

Figure 2.1(a) shows the schematic diagram of the single RBCs biophysical characterization system. The microfluidic chip was constructed by bonding PDMS microchannels on a glass slide. Two Ag/AgCl non-polarizable electrodes connected to the function generator (sinusoidal voltage at 100 kHz @1.0 V_{pp}) and the lock-in amplifier (SR850, Stanford Research Instruments, USA) were inserted into the inlet and outlet ports of the microfluidic device. The analog outputs of the lock-in amplifier were sampled with a 16-bit DAQ card (NI PCI-6229, National Instruments, USA) and data capture software (LabVIEW, National Instruments, USA). Dilute blood sample was pipetted into the inlet reservoir of the device and driven through the constriction channel by hydraulic pressure difference (see Figure 2.1(b)).

Figure 2.1(c) shows the equivalent circuit model. The channel is equivalent to a resistor $R_{\text{channel}}$ and a capacitor $C_{\text{channel}}$ in parallel [64]. As RBCs (including membrane capacitance $C_{\text{cell}}$ and cytoplasm resistance $R_{\text{cell}}$) [100, 117] pass through the constriction channel, they perturb the electric field in the volume within the channel generating a current impulse. $R_{\text{leak}}$ presents the sealing resistance between the RBC membrane and channel walls. In the mean time, the current change of the circuit loop is sensed via input impedance ($I_{\text{inner}}$) of the lock-in amplifier (10 MΩ+25 pF), amplified and recorded by the data capture software. Outputs of the lock-in amplifier consist of the real component ($X$) and the imaginary component ($Y$). The amplitude ($A$) and phase ($\Phi$) were calculated according to $A = \sqrt{X^2 + Y^2}$, $\Phi = \arctan\left(\frac{Y}{X}\right)$. A processing algorism was used to extract the transit time (the time duration taken by a cell to travel through the constriction channel, $\Delta T$), the amplitude ratio (the ratio between the lowest amplitude value captured during cell’s squeezing through the constriction
channel and the amplitude value with no cell in the constriction channel, \((A - \Delta A)/A\), and the phase difference between with cells and without cells, \((\Delta \Phi)\) were quantified as RBCs’ mechanical and electrical property indicators.

Compared with previously reported microfluidic devices for biophysical measurements, this design has a few advantages. Firstly, almost all electrical field lines are forced to penetrate RBCs’ membrane and hemoglobin inside, making the device more sensitive to minute biophysical differences. Secondly, our measurement is purely electrical, eliminating the need for microscopy imaging and hence, permitting high measurement throughputs. High-speed cameras (tens of kHz) generate gigabyte data per second and can only record for a few seconds due to limited on-board memory. Processing this massive amount of image data also takes tremendous computation efforts and time. Therefore, for microfluidic single cell measurement systems, high-speed imaging offers high speeds, but total number of data points (i.e., sample size) is limited [45, 138]. Thirdly, no sheath flow is required on our device. Since the constriction channel’s cross-sectional area is smaller than the diameter of RBCs, only a single RBC is permitted to pass through the constriction channel in a given time instance simply by tuning the density of the RBC suspension.
CHAPTER 2, HIGH-THROUGHPUT BIOPHYSICAL MEASUREMENT

Figure 2.2 Experimental amplitude profiles: (a) a single RBC, (b) two RBCs, (c) a single platelet, and (d) a single WBC within the constriction channel.

2.3 Materials and methods

2.3.1 Device fabrication

The constriction channel (first layer) was fabricated with SU8-2002 (5μm×3μm) (MicroChem Corp., Newton, MA, USA) on a glass slide. A second layer of SU8-25 was then spin coated on the glass slide covered with the first layer features, soft-baked, and exposed to UV light with alignment, followed by post-exposure bake, development and hardbake. The
loading channel’s cross-sectional area (1000μm×30μm) is much larger than that of the constriction channel. Hence, the impedance of the device is mainly determined by the constriction channel. The microchannels were molded with PDMS (Ellsworth Adhesives, ON, Canada), punched to form inlet and outlet ports, and bonded to a glass slide treated with a corona-treater (Electro-Technic Products Inc., Illinois, USA).

2.3.2 Blood samples and experimental protocol

Peripheral blood samples were obtained following routine blood tests at the hospital hematology laboratory. Blood samples were collected using commercial vacuum tubes with EDTA anticoagulant (ethylenediaminetetraacetic acid 1.5mg/ml) (Sigma-Aldrich, Oakville, ON, Canada). The adult (n=5) and newborn samples (n=5) were of normal individuals with the complete blood counts performed by a standard commercial hematology analyzer (Sysmex XE-2100, Kobe, Japan). In the experiments, 10μL blood was diluted in 500 μL PBS (Sigma-Aldrich, Oakville, ON, Canada) mixed with 0.2% w/v Pluronic (Sigma-Aldrich, Oakville, ON, Canada) and 1% w/v BSA (New England Biolabs Inc., Herts, UK). Pluronic and BSA were used for preventing non-specific adhesion to the channel walls. The dilute samples were placed for 20 minutes before usage. Before the sample was pipetted into inlet port of the device, the microchannel was filled with PBS solution with 0.2% w/v Pluronic and 1% w/v BSA. A pressure difference (50 Pa) between the inlet and outlet ports was applied for 30 minutes to ensure the equilibrium in lubrication of the channel walls. A 5 μL dilute blood suspension was then pipetted into the entrance of the cell loading channel. The two Ag/AgCl non-polarizable electrodes were inserted into the inlet and outlet ports of the device. A negative pressure of 3 kPa was then applied to aspirate cells continuously through the constriction channel while electrical data was sampled.
2.3.3 Electrical measurement and data analysis

A sinusoidal voltage (100 kHz @1.0 V_{pp}) was applied to the two Ag/AgCl electrodes. When an RBC is aspirated into the constriction channel, it blocks electric field lines and causes the current in the circuit loop to drop. During experiments, the real component (X) and the imaginary component (Y) of the lock-in amplifier output were sampled at 120 kHz. X and Y were converted to amplitude (A) and phase (Φ). Figure 2.2 shows amplitude profiles of a single RBC, two RBCs, a single platelet, and a single WBC (white blood cell) within the constriction channel.

The throughput of our system is 100-150 cells/second. The variation in throughput depends on cell density differences across patient samples. A threshold is defined as 98% of the basal amplitude (the amplitude without cell presence in the constriction channel) (see Figure 2.2(a)). Comparing a signal and the threshold amplitude value, the portions where the signal’s amplitude is lower than the threshold value were considered as cell passage regions. A quadratic polynomial peak detector was used to detect the valleys within the cell passage regions. More than one valley within a cell passage region suggests the passage of more than one cell (see Figure 2.2(b)). The time period between the two intercepts with the threshold value was interpreted as cell transit time (ΔT), which is determined by the cell’s size and mechanical stiffness. The amplitude ratio ((A-ΔA)/A) (see Figure 2.2 (a)) and the phase increase (ΔΦ) were quantified as the cell’s electrical signatures. Since the electrical field lines are highly concentrated by the constriction channel to penetrate the cell, the electrical signal generated by a single RBC in our experiments was readily distinguishable from the background noise (SNR≥29dB). No preamplifier and additional filters were required.
CHAPTER 2, HIGH-THROUGHPUT BIOPHYSICAL MEASUREMENT

Figure 2.3 Ratio of the amplitude change and the basal amplitude vs. aspiration pressure. Device sensitivity becomes lower at higher aspiration pressure due to RBC deformation and larger leakage current.

Figure 2.4 Histograms of transit time, amplitude ratio and phase increase attained from an adult sample (22,669 cells measured), (red)(cyan) before and after the exclusion of platelets and debris.
Figure 2.5 Adult RBCs (n=84,073 from 5 adult samples, red), neonatal RBCs (n=82,253 from 5 newborn samples, cyan). (a) 3D scatter plot of transit time vs. amplitude ratio vs. phase increase. (b) Histograms of transit time, amplitude ratio, and phase increase fitted with normal distributions.
2.4 Results and discussion

2.4.1 Selection of channel dimension, signal frequency, and applied pressure

Constriction channels having a smaller cross-sectional area can form a better seal between the RBC membrane and the channel walls. However, RBCs collapsed when the cross-sectional area was much smaller than RBCs’ diameter. For constriction channels with a cross-sectional area of 1µm×3µm, almost 100% RBCs collapsed at the entrance of the constriction channel. For constriction channels with a cross-sectional area of 2µm×5µm, the majority of RBCs were elongated and then collapsed within the constriction channel. For constriction channels with a cross-sectional area of 3µm×5µm (chosen for our experiments), all RBCs showed folded morphology, and no RBCs collapsed.

RBCs are highly deformable. The cell shape in the constriction channel is altered by fluid shear stress. At higher aspiration pressures, RBCs are further deformed in the shear stress direction, resulting in a poorer seal between the RBC membrane and constriction channel walls. Consequently, signal change with and without cell’s presence in the constriction channel becomes smaller due to larger leakage current. Figure 2.3 shows the ratio of the amplitude change and the basal amplitude, measured on the same blood sample at different aspiration pressures. Each data point in the figure is from approximately 1,000 cells. In our subsequent experiments, 3 kPa was chosen for a balance between throughput and detection sensitivity. At this aspiration pressure, the velocity of the fluid in the constriction channel is approximately 30mm/sec. As to frequency selection, the frequency should be high enough to enable the electrical field lines to penetrate the RBC membrane such that the electrical property of RBCs can be reflected by the measured electrical signal. Additionally, the frequency should not be too high. Too high a frequency would result in extremely low
impedance of $C_{\text{channel}}$ and make electrical field lines undesirably obviate the cell in the constriction channel [32, 64]. We experimentally selected 100 kHz for subsequent experiments.

### 2.4.2 WBCs and platelets

Although RBCs represent over 90% blood cells, white blood cells (WBCs) and platelets remained in the sample. WBCs and platelets were excluded in data processing. As shown in Figure 2.2, electrical signals measured from RBCs, WBCs, and platelets are drastically different, thus using amplitude ratio alone proved highly effective to distinguish WBCs and platelets from RBCs.

Figure 2.4 shows histograms attained from an adult blood sample (22,669 cells measured). The red colored histograms show data after the exclusion of WBCs. The relatively small population (circled) within the blood sample barely perturbed the electrical field lines. This population was made up of platelets and debris that have diameters smaller than 3μm. The “cyan” data in Figure 2.4 are transit time, amplitude ratio, and phase increase with fitted normal distributions after excluding platelets and debris.

### 2.4.3 RBC measurements

Our microfluidic system (frequency: 100 kHz, pressure difference: 3 kPa, constriction cross-section area: $3\mu\text{m} \times 5\mu\text{m}$) tested adult RBCs (5 samples, ~16,000 cells/sample) and neonatal RBCs (5 samples, ~16,000 cells/sample). As discussed earlier, WBCs and platelets were easily distinguished from RBCs using their distinct amplitude ratio. Additionally, the occurrence of two or more RBCs (vs. single RBCs) inside the constriction channel at the same time was rather rare (<5%). Figure 2.5(a) shows the 3D scatter plot of transit time vs.
amplitude ratio vs. phase increase of all adult RBCs (n=84,073 from 5 adult samples) and neonatal RBCs (n=82,253 from 5 newborn samples). The ellipses in the figure track the standard deviation of the distribution. Each of the three parameters was identified and fitted to normal distribution profiles (see Figure 2.5(b)). A back propagation neural network was used for pattern recognition (MATLAB, MathWork, USA). The input data have three groups of parameters (transit time, amplitude ratio, and phase increase) measured on RBCs. The complete dataset was divided into training data (70%), validation data (15%), and testing data (15%) to quantify adult vs. neonatal RBC classification success rates (i.e., accuracy). RBC classification success rates were 76.2% (transit time + amplitude ratio), 78.1% (amplitude ratio + phase increase), 77.9% (phase increase + transit time), and 84.8% (amplitude ratio + phase increase + transit time), suggesting multiple parameters (transit time, amplitude ratio and phase increase), when used in combination, can provide a higher cell classification success rate. Besides the success rate of 84.8%, sensitivity (true positive/(true positive + false negative)) and specificity (true negative/(true negative + false positive)) were 80.2% and 89.2%, respectively.

Cell transit time is determined by RBCs’ volume, membrane stiffness, and the friction between the membrane and channel walls. The electrical impedance amplitude ratio and phase increase are determined by RBCs’ volume, ion channels on the membrane, and the density of hemoglobin. Hence, transit time, amplitude ratio, and phase increase are not completely independent parameters (e.g., they are all affected by cell volume). The neural network classification results also indicate that each of the three parameters can reflect unique properties of RBCs, leading to higher classification success rates when used in combination.
The amplitude change during a cell’s passage through the constriction channel is caused by the blockage of the electrical field lines. Figure 2.6(a) reveals a linear trend between the amplitude ratio and the cell volume for both adult RBCs and neonatal RBCs with different slopes (-0.0024 vs. -0.00356). Cell electrical properties are determined by membrane capacitance and cytoplasm (hemoglobin in the case of RBCs) resistance. Since the lipid layer composition of neonatal RBCs and adult RBCs is very similar [139] and the effective area of the membrane capacitance is constricted by the cross section of the constriction channel, the different slope indicates the different conductivity between neonatal RBCs and adult RBCs. This can possibly be related to the higher hemoglobin density inside neonatal RBCs. The average RBC volume and hemoglobin density of each sample were obtained from complete blood counts performed by a commercial hematology analyzer.

As shown in Figure 2.6 (b), transit time as a function of RBC volume can be fitted into a single line for both adult RBCs and neonatal RBCs. The transit time of RBCs under a constant negative pressure is determined by the friction force between the membrane and channel walls (friction force = contact pressure × contact area × friction coefficient, where contact pressure is related to membrane stiffness and friction coefficient is related to membrane composition). It is known that the lipid layer composition and membrane stiffness of neonatal and adult RBCs are very close [139, 140]. Hence, contact pressure and friction coefficient should be comparable for adult and neonatal RBCs. In the constriction channel, contact area is proportional to RBC volume. Therefore, it is not surprising that transit time as a function of RBC volume can be fitted into a single line for both adult and neonatal, indicating that the transit time difference for adult RBCs and neonatal RBCs were mainly caused by their volume difference.
Figure 2.6 (a) Amplitude ratio as a function of RBC volume. (b) Transit time as a function of RBC volume.
2.5 Conclusion

This chapter presented a microfluidic system capable of measuring multiple biophysical parameters on single RBCs. Compared with previously reported microfluidic devices for single RBC biophysical measurement, this system has a higher throughput (100-150 cells/second), higher signal to noise ratio, and the capability of performing multi-parameter measurements. The microfluidic system may have potential applications in drug efficacy testing and RBC property characterization relevant to clinical conditions. Pattern recognition confirmed that a combination of measurements of transit time, electrical impedance amplitude, and impedance phase resulted in a high success rate in classifying fetal/neonatal and adult RBCs. However, the achieved 89.2% specificity and 80.2% sensitivity for cell classification require further improvement for diagnostics applications such as rare fetal RBC enumeration in adult blood.
Chapter 3

3. Electrical Measurement of Red Blood Cell Deformability on a Microfluidic Device

3.1 Introduction

Red blood cells (RBCs) are highly deformable, allowing them to be able to travel through in-vivo capillaries with diameters smaller than RBCs’ size, which facilitates gas transfer between blood and tissues [17, 141, 142]. Decrease in RBC deformability can disturb blood flow and oxygen delivery. Essentially, the deformability of RBCs is determined by the integrity and organization of the membrane cytoskeletal protein network and density, and the viscosity of the cytoplasm. Pathological condition changes may lead to significant alteration and reorganization of the protein networks, and consequently compromise RBCs’ deformability [7, 17, 143]. For example, the polymerized and deoxygenated hemoglobin in sickled RBCs causes deformability to decrease [144]. In the case of malaria, the impaired deformability of RBCs is in association with the interruption of the cytoskeleton network by parasite invasion [145]. In addition to an indicator for pathological states, RBC deformability can also serve as a criterion for stored/banked blood quality assessment [146, 147].
Several standard tools can be applied to measuring RBC deformability. Atomic force microscopy (AFM) assesses the deformability of a cell by indenting/deforming the cell through physical contact [13, 148]. Micropipette aspiration applies a negative pressure through a glass micropipette to aspirate a cell patch for characterizing the cell’s mechanical parameters [13, 63]. Optical tweezers stretch a cell by optically manipulating high-refractive-index dielectric beads attached to the cell membrane [4, 13]. These standard tools involve high operation complexity and have a limited testing speed of minutes per cell.

Hence, other techniques were developed for testing RBC deformability with improved speed and/or reduced operation complexity [12, 133, 149]. When RBCs flow through capillaries, they are deformed by shear stress into a parachute-like shape [141, 150]. Accordingly, microfluidic channels were used to mimic human capillaries and study RBC deformability. Shear stress-induced cell deformation was captured via a high speed camera [35, 142, 151]. The use of high-speed cameras also necessitates a microscope and a high intensity lighting system, making the system inevitably bulky and costly. Furthermore, present high-speed cameras have limited on-board memory, permitting only a few seconds’ recording and thus, limiting testing throughput (number of cells per test). Post-processing of tens of GB image data is also time-consuming.

Cell Transit Analyzer (CTA) consists of micro pores that are smaller than RBCs. It measures cell transit time (i.e., the time required by an RBC to pass through) via electrical resistance changes or high-speed imaging. This approach is able to provide an increased throughput and is capable of performing multi-parameter measurements, particularly when electrical measurement is used (100-150 cells/sec) [23]. Recently, the CTA approach has been adapted for assessing the deformability of other cell types, including human cancer cells.
[31-33]. An important advantage of CTA is its simplicity [20]; however, cell volume variations and adhesion between the cell surface and channel walls are coupled with cell deformability, which together determine cell transit time. In other words, cell transit time is not only determined by cell deformability but also the volume/size and membrane properties.

In ektacytometry, RBCs are deformed by shear stress, and laser diffraction patterns of RBCs are analyzed [152, 153]. Diluted blood is loaded into a small gap between two rotating plates. RBCs are allowed to settle for a period of time to form weak adhesion with the bottom surface of the test chamber. Shear stress (e.g., 0.5-50 Pa) is controlled by varying the rotation rate of the plates. Elongation index of the RBCs is measured using laser diffractometry. Ektacytometers are relatively easy-to-use; however, the measurement principle is difficult to implement on a miniaturized device design. Importantly, different from CTA wherein RBCs flow continuously, in ektacytometry, the throughput is determined by the imaging field of the view. This limits testing throughput to approximately 50-60 RBCs per test (LoRRca MaxSis user manual, Mechatronics Manufacturing B.V., Netherlands).

Due to the simplicity of electrical measurement, measuring RBC deformability electrically was attempted [154]. In this reported system, medium viscosity was modified to achieve sufficient shear stress. It was incapable of decoupling RBC volume/size from deformability. Thus, RBCs with larger diameters can be mistakenly determined to be more deformable. This chapter presents a microfluidic system for electrically measuring RBC deformability. The microfluidic device has two measurement stages, with cross-sectional areas of 8 µm × 8 µm and 5 µm × 5 µm, respectively. RBCs are pressure driven to flow through the microfluidic channels while electrical current changes are measured via two
Ag/AgCl electrodes. To mitigate the coupled effect from cell volume, *mechanical opacity* is defined as an indicator of RBC deformability.

Figure 3.1 Electrical measurement of RBC deformability. The schematics show two RBCs with identical size/volume but different deformability. A voltage signal is applied across the channel, and current (denoted as yellow arrows) is measured via a current preamplifier. Shear stress induces the RBCs into a parachute-like shape. The RBC with higher deformability is more stretched along the flow direction (a), resulting in larger gaps between the RBC membrane and channel walls than the less deformable RBC (b). Thus, the RBC with higher deformability blocks less current (i.e., smaller current decrease in the current profile) than the RBC with lower deformability.
Figure 3.2 (a) Microfluidic device for electrically measuring RBC size and deformability. Ag/AgCl electrodes are plugged into the inlet and outlet ports via T-junctions. The cross-sectional areas of the measurement units are 8 µm × 8 µm and 5 µm × 5 µm, respectively. The transit region (50 µm × 20 µm) is for separating the two current valleys of the two measurement units. RBCs are driven through the measurement areas continuously. (b) Experimental data: time series of the current profile measured within 1 second with 14 RBCs passing through. (c) The zoom-in view of the profile circled in (b).
3.2 Measurement Principle

Under shear stress, RBCs change into a parachute-like shape. The cell is stretched along the flow direction, resulting in gaps between RBC membrane and microchannel walls [141, 151, 155-157]. As shown in Figure 3.1, a voltage signal (10 kHz@0.5 V_{pp}) is applied across the microfluidic channel. RBCs have a specific membrane capacitance value of 9 ± 0.8 mF/m\(^2\), and hence act as an insulated layer at low frequencies (e.g., 10 kHz) [33, 158, 159]. When an RBC is inside the channel, a portion of the conductive medium/liquid is replaced. Thus, only the conductive medium within the gaps between the cell membrane and channel walls conducts current, which causes the current passing through the channel to decrease. For two RBCs have the same volume/size but different deformability, the more deformable RBC [Figure 3.1 (a)] is stretched more along the flow direction leaving larger gaps between the cell membrane and channel walls (vs. the less deformable RBC). Therefore, electrical current within in the channel is less blocked (vs. [Figure 3.1 (b)]), and in the measured current profile, current decrease is smaller. In other words, current decrease in this case is correlated to RBC deformation. However, in addition to deformability, current decrease is also dependent on RBC size/volume, since a larger volume replaces a larger volume of conductive medium, and thus causes stronger current blockage.

In order to take the cell volume effect into account, our device design has two measurement units [Figure 3.2 (a)]. Two Ag/AgCl electrodes are inserted into the inlet and outlet ports via T-junctions to apply a voltage signal and measure current changes. Custom-made water tanks were used to generate pressure for driving RBCs through the microchannels. The cross-sectional area of the first measurement unit is 8 µm × 8 µm, and the cross-sectional area of the second measurement unit is 5 µm × 5 µm. The transit region in
between (50 µm × 20 µm) is designed for separating the two current valleys [Figure 3.2 (b)(c)] of the two measurement units for the convenience of signal processing. Figure 3.2 (b) shows experimental data that are time series of the current profile measured within 1 second with 14 RBCs passing through the measurement area with a pressure difference of 1600 Pa. The zoom-in view of the profile circled in Figure 3.2 (b) is presented in Figure 3.2 (c). The small valley (I₁) of the current profile corresponds to the current decrease when an RBC passes through the 8 µm × 8 µm channel; and the large valley (I₂) was generated when the same RBC passed through the 5 µm × 5 µm channel.

As discussed earlier in this section, the electrical current signals from both 5 µm × 5 µm channel and 8 µm × 8 µm channel are dependent on cell volume and deformation. The shear stress within the 5 µm × 5 µm channel is approximately four times as high as that in the 8 µm × 8 µm channel. As a result, RBCs are much more deformed in the 5 µm × 5 µm channel. Additionally, due to the smaller cross-sectional area, current signal is more sensitive to changes of gaps between the cell membrane and channel walls in the 5 µm × 5 µm channel. In the meanwhile, the current signal generated in the 8 µm × 8 µm channel is more reflective of the RBCs volume information, as the Coulter counter principle [71, 160]. To mitigate the coupled effect from cell volume and cell deformability, the ratio of I₂/I₁ is defined in this study as mechanical opacity, and is used as a measure of RBC deformability.

3.3 Materials and Methods

3.3.1 Blood sample preparation

Whole blood samples were obtained from healthy donors (Mount Sinai Hospital, Toronto, Canada). Blood samples were anticoagulated with EDTA anticoagulant
(ethylenediaminetetraacetic acid 1.5 mg/ml) (SigmaAldrich, Oakville, ON, Canada) and washed twice with PBS (Sigma-Aldrich, Oakville, ON, Canada) before further treatment. Glutaraldehyde (GA)-treated and heated RBCs have been commonly used in the literature for studying RBC deformability. GA is a nonspecific fixative that lowers RBC deformability by cross-linking the cytoskeletal proteins [35]. Heating can significantly denature the RBC cytoskeletal network and make RBCs more deformable [161]. For glutaraldehyde (GA) treated samples, the washed RBCs were suspended into PBS added with 0.005 % glutaraldehyde, and incubated at room temperature for 30 min. Following incubation with GA, the RBCs were washed three times with PBS. For heat treatment, the washed RBCs were re-suspended in a PBS solution and heated in a water bath maintained at 49 °C for 30 min. Thereafter, all RBCs samples were suspended in PBS with 1% w/v BSA with hematocrit of 0.45%, and incubated for 30 min at room temperature to prevent adhesion.

3.3.2 Device fabrication and operation

The device was fabricated using standard multilayer soft lithography [32, 162]. The microfluidic channel consists of four different cross-section areas: two measurement units (5 µm × 5 µm and 8 µm × 8 µm); the transit unit (50 µm × 20 µm) in between the two measurement units; and the loading channel (500 µm × 80 µm) for loading RBCs to the measurement areas. Since the loading channel’s cross-sectional area is much larger than the measurement units, electrical current is largely determined by the resistance of the measurement areas. Before experiments, the microchannel was incubated with PBS (Sigma-Aldrich, Oakville, ON, Canada) mixed with 0.2% w/v Pluronic (SigmaAldrich, Oakville, ON, Canada) and 1% w/v BSA (New England Biolabs Inc., Herts, UK) for 30 min. A droplet of RBC sample was pipetted into the inlet port of the device. The two T-junctions with
Ag/AgCl non-polarizable electrodes were then inserted. The T-junctions were connected with custom-made water tanks for accurately generating pressure differences.

3.3.3 Signal processing

A sinusoidal voltage signal (10 kHz@0.5 V_{pp}) was applied through the Ag/AgCl electrodes. A current preamplifier connected in series into the current pathway, amplified and converted current into voltage signals, which is sampled by a computer. When RBCs flow through the measurement units, current (RMS) is measured continuously, as shown in Figure 3.2 (b)(c). At low frequencies, the electrical current signal can better reflect RBCs deformation since current cannot penetrate the RBC membrane. The frequency of 10 kHz was chosen as a compromise of system time response and deformation measurement sensitivity. Experimental data was processed using a custom-built program. Briefly, basal current was extracted from the raw data using a histogram-based technique. Peak threshold values were calculated as 98% of the basal current. Peak detection was conducted after noise filtering. The blood samples used in experiments were from whole blood and contained white blood cells (WBCs) and platelets. In the data processing, WBCs and platelets were excluded from the final results on the basis of distinct current signals. Data processing also excluded the rare events with multiple cells within the measurement unit, where consecutive large valleys appear in the measured data.
Figure 3.3 Current decrease within 8 µm × 8 µm measurement unit (I₁, red) and 5 µm × 5 µm measurement unit (I₂, blue) of a controlled RBC sample under various driving pressures. The error bars represent standard deviation. More than 1,000 RBCs were measured for each data point.

3.4 Results and Discussion

3.4.1 Shear stress effect

The pressure difference generated with the water tanks ranged from 400 Pa to 1600 Pa. The corresponding average shear stress in the 5 µm × 5 µm channel was approximately 8 Pa to 36 Pa. This pressure range was tested to make a comparison with the results from commercial ektacytometers (shear stress range 0.5-50 Pa). A controlled RBC sample was tested under several pressures (400Pa, 700 Pa, 1000Pa, 1300 Pa, 1600Pa). The electrical current decrease
of the 5 µm × 5 µm channel (I₂, blue) and 8 µm × 8 µm channel (I₁, red) under these pressures is summarized in Figure 3.3. When an RBC is in the 5 µm × 5 µm channel, current change is highly sensitive to pressure since under increasing pressures, the RBC is further stretched along the flow direction. While in the 8 µm × 8 µm channel, shear stress is lower, and current change is far less sensitive to pressure variations.

Ideally, the first measurement unit should be much larger than the size of RBCs so that the low shear stress hardly deforms a cell, and the current signal is only determined by cell volume/size. However, too large a cross-sectional area in the Coulter counter measurement unit in experiments caused too small a current change when a cell passed through. Therefore, 8 µm × 8 µm was chosen to achieve a reasonable signal-to-noise ratio and reduce the cell deformation effect on the measured signal. As shown in Figure 3.3, although the measured signal shows slight changes as the applied pressure increases, the change is much less significant than the signal generated in the 5 µm × 5 µm channel. In the 5 µm × 5 µm channel, current signal is determined by the gaps between RBC membrane and the channel walls. However, it is challenging to experimentally measure the gaps using standard microscope imaging.

3.4.2 Mechanical opacity

Controlled, GA-treated, and heated RBCs were tested using this system. The scatter plots show I₂ versus I₁ for controlled RBCs and GA-treated RBCs [Figure 3.4 (a)], and controlled RBCs and heated RBCs [Figure 3.4 (b)]. The driving pressure was 400 Pa. In Figure 3.4 (a)(b), I₂ shows a strong linear correlation with I₁. Comparing the controlled RBCs and GA-treated RBCs with the same I₁ value (volume indicator), the GA-treated RBCs have clearly higher I₂ values, reflecting the fact that the controlled RBCs are more deformable than the
GA-treated RBCs because they are stretched more along the flow direction (larger gaps). The heated RBCs reveal a lower $I_2$, indicating heated RBCs are more deformable than controlled RBCs. Here, we borrow the concept of impedance opacity (i.e., ratio of impedance at high frequency to low frequency) from impedance flow cytometry [116, 117]. Due to the strong linear dependence, the ratio of $I_2/I_1$ was thereby used to normalize RBC volume, which is defined as mechanical opacity. Based on the mechanical opacity that reflects RBC deformability, GA-treated RBCs and heated RBCs can be effectively separated from controlled RBCs [Figure 3.4 (c)].
Figure 3.4 (a) Scatter plot of $I_2$ vs. $I_1$ for controlled RBCs ($n=996$) and GA-treated RBCs ($n=1,059$). (b) Scatter plot of $I_2$ vs. $I_1$ for controlled RBCs ($n=996$) and heated RBCs ($n=1,179$). All RBCs were measured under a driving pressure of 400 Pa. $I_2$ depends linearly on $I_1$. For RBCs with the same $I_1$, GA-treated RBCs and heated RBCs show a higher and lower $I_2$ than controlled RBCs, respectively. (c) Scatter plot of $I_2/I_1$ (opacity) vs. $I_1$ for controlled, GA-treated and heated RBCs. RBCs deformability is reflected by the ratio of $I_2/I_1$. The data show that GA treatment decreases RBC deformability and heat treatment increases RBC deformability.
3.4.3 System characterization

Figure 3.5 (a)(b) shows the histograms of $I_2/I_1$ (opacity) of controlled, GA-treated, and heated RBCs measured under 400 Pa and 1600 Pa, fitted with normalized distribution. These two pressures were chosen as representatives of low and high pressure. As expected, the less deformable population (GA-treated) shows an opacity distribution shifted to the right, and the more deformable population (heated) shows an left shifted opacity distribution, compared to the controlled population. It was also found that the GA-treated RBCs have a wider distribution compared to controlled and heated RBCs. As driving pressure increases [Figure 3.5 (b)], the difference in the average opacity values among the populations becomes smaller, implying lower sensitivity for distinguishing the three populations. Figure 3.5 (c) summarizes the ratio of $I_2/I_1$ (opacity) of controlled RBCs (red), GA-treated RBCs (blue), and heated RBCs (green) measured under different driving pressures. The GA-treated RBCs (less deformable) shows consistently higher mechanical opacity than controlled RBCs while heated RBCs consistently have lower mechanical opacity values (more deformable). As driving pressure increased, the difference between different populations became smaller. P-values of the two-group T-test show statistically significant difference between the controlled and treated RBCs at all applied pressure ($p \ll 0.05$).

To evaluate the power to detect differences between controlled and treated RBCs, standardized difference values in comparison with controlled RBCs were calculated according to

$$SD = \frac{(X_1 - X_2)}{\sqrt{(S_1^2 + S_2^2)/2}}$$
where \(X_1\), \(X_2\) and \(S_1\), \(S_2\) denote the sample mean, standard deviation of each group, respectively.

As shown in Figure 3.5 (d), standardized difference values of both GA-treated and heated RBCs decrease as the driving pressure increases. This finding is in agreement with the results generated using ektacytometry \([163, 164]\). Ektacytometry results showed that RBC deformation became less sensitive as shear stress increased from several Pa to 30 Pa, which caused lower standardized difference values.

We further investigated the effect of RBC volume on the measured mechanical opacity. Figure 3.6 (a)(b) shows the average and standard deviation of measured mechanical opacity for different ranges of \(I_1\), under 400 Pa and 1600 Pa, respectively. Since \(I_1\) is an indicator of RBC volume, comparing opacity values within each \(I_1\) sub-range could reveal the deformability of RBCs within the same size group. For each RBC population (controlled, GA-treated, and heated), the mechanical opacity values across the four size (\(I_1\)) sub-ranges are all close to the population’s overall average value; the standard deviation of all controlled, GA-treated and heated RBCs show very little variation across the four sub-ranges. These results indicate that the mechanical opacity quantity mitigates the coupled effect from RBC volume and reflects RBC deformability.

### 3.5 Conclusion

This chapter presented a microfluidic system for electrically measuring RBC deformability. The two-stage device design and measurement technique together mitigate the cell volume effect on RBC deformability. By measuring controlled, GA-treated and heated RBCs, the system demonstrated the capability of distinguishing RBC populations having known
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dehormability differences. The electrical measurement system has a speed of 10-20 cells per second. The speed can be further improved by increasing cell density and driving pressure. However, as shown in Figure 3.5, the ability to distinguish different populations would decrease at higher driving pressures.
Figure 3.5 Histograms of $I_2/I_1$ (opacity) of controlled, GA-treated and heated RBCs under (a) 400 Pa and (b) 1600 Pa driving pressures, fitted with normalized distribution. (c) Mechanical opacity of controlled RBCs (red), GA-treated RBCs (blue) and heated RBCs (green) measured under different driving pressures. Results are mean ± standard deviation. More than 1,000 RBCs were tested for each of the data points. (d) Standardized difference values calculated using the data present in (c). Blue: standardized difference between GA-treated and controlled RBCs. Green: standardized difference between heated and controlled RBCs.
Figure 3.6 Comparison of $I_2/I_1$ (opacity) of controlled, GA-treated, and heated RBCs within sub-ranges of $I_1$. (a) 400 Pa driving pressure, (b) 1600 Pa driving pressure. Error bars represent standard deviation.
Chapter 4

4. Microfluidic Characterization of Specific Membrane Capacitance and Cytoplasm Conductivity of Single Cells

4.1 Introduction

Each cell type has unique membrane contents and intracellular structures to fulfill their specific physiological functions. The membrane and intracellular compositions determine the cell’s electrical properties that can reflect cell physiological states and can possibly serve as label-free markers for cell type classification. Compared to conventional biochemical techniques (e.g., flow cytometry), which rely on the labeling of targeted cells with fluorescence-conjugated antibodies or molecules to distinguish between cell types, electrical measurement has distinct simplicity in sample preparation (no labeling), and in data recording and processing [100, 165, 166].

In order to interpret the electrical properties of a cell, a single shell model is widely used in various measurement setups [79, 80]. The cell is modeled as a dielectric thin membrane enclosing homogenous spherical cytoplasm. Accordingly, the specific membrane capacitance (affected by the membrane morphology, lipid bilayer composition and thickness, and
embedded proteins [81-83]) and cytoplasm conductivity (influenced by the nucleus cytoplasm ratio and the ion concentration inside the cell [84-86]) are depicted as the inherent electrical properties of the cells’ membrane and cytoplasm, respectively. In the past decades, a number of techniques were developed for single-cell electrical measurements. However, existing technologies are either incapable of characterizing the inherent electrical properties or incapable of providing statistically significant data due to limited measurement speed.

The most successful technique performing electrical measurement on single cells is the Coulter counter, where direct current (DC) impedance measurements are applied for counting and sizing single cells [167]. Impedance flow cytometry is an extension of the Coulter counter technique. It implements both DC and RF impedance measurements. The ratio of the RF signal to DC signal is defined as opacity, which is applied in commercial hematology analyzers for three differential counts of white blood cells [168, 169]. However, the opacity value reflects combined effects from both cell membrane and cytoplasm. It is not an inherent electrical signature of cells and can differ among different testing configurations (e.g., co-planar electrodes vs. parallel facing electrodes) [100, 119]. µ-EIS (Micro Electrical Impedance Spectroscopy) is a technique in which a frequency-dependent excitation signal is applied across a trapped cell to measure the corresponding current response [108, 110, 111]. Similar to opacity, capacitance and resistance of a cell are not inherent electrical parameters (vs. specific membrane capacitance and cytoplasm conductivity) and are strongly affected by electrode size, cell trapping mechanism, and cell volume.

The patch-clamp technique was originally developed for measuring current through ion channels on the cell membrane, and can be used for characterizing the specific membrane capacitance by aspirating a cell membrane patch into a micropipette [170, 171]. However, it
is a highly delicate procedure and has strong operator skill dependence. Consequently, the testing speed of patch-clamp is typically tens of minutes per cell, and at most tens of cells can be tested in a study. Electrorotation is capable of quantifying electrical properties of single cells by measuring the rotation rate of the targeted cells induced by the rotating electrical field (e.g., specific membrane capacitance, cytoplasm conductivity and cytoplasm permittivity). Nonetheless, electrorotation is also a rather tedious and slow technique, and it typically takes approximately 30 minutes to test a single cell [101, 105]. Another drawback is the difficulty to achieve efficient rotation in high conductivity physiological buffer. Hence, electrical properties of the tested cells may have already been altered when immersed in the low conductivity sucrose buffer. At present, no techniques exist for measuring the specific membrane capacitance and cytoplasm conductivity values at a reasonable speed.

This chapter presents a new technique for measuring single-cell specific membrane capacitance and cytoplasm conductivity at a speed of 5-10 cells/second. A microfluidic constriction channel that is marginally smaller than the diameters of tested cells concentrates the electrical current to penetrate the cell membrane and confines the geometry of individual cells. Electrical impedance was measured at seven frequencies simultaneously. By fitting experimental impedance measurement data to equivalent circuit models, the specific membrane capacitance and cytoplasm conductivity values of over 6,000 cells were quantified. AML-2 and HL-60 cells were selected for their comparable sizes to assess the effectiveness of distinguishing these size-comparable cell types using their electrical parameters. Compared to the impedance measurement system we previously reported [32], the new multi-frequency measurement technique and the improved equivalent circuit model with cell geometry estimation enable, for the first time, the determination of the specific membrane
capacitance and cytoplasm conductivity values on a high number of cells (3,249 AML-2 cells and 3,398 HL-60 cells).

4.2 System overview

Figure 4.1 (a) shows the schematic diagram of the single cell electrical measurement system. The microfluidic chip was constructed by bonding PDMS microchannels to a glass slide. Cell suspension was pipetted into the inlet reservoir of the device and driven through the constriction channel by hydraulic pressure difference (500 Pa). Two Ag/AgCl non-polarizable electrodes are plugged into the inlet and outlet ports. A sinusoidal excitation voltage with 7 frequency components (1 kHz, 20 kHz, 50 kHz, 100 kHz, 200 kHz, 300 kHz, 400 kHz, @0.2 V_{pp}) is generated by the function generator. As cells are aspirated through the constriction channel continuously, current within the channel is pre-amplified, demodulated and sampled at 14.4 kHz per frequency. By fitting the impedance spectroscopy (7 frequencies) to the equivalent circuit models, membrane capacitance (C_{mem}) and cytoplasm resistance (R_{cyto}) can be obtained. Within the constriction channel, the cells’ shape is well confined; hence, the C_{mem} and R_{cyto} values can be used to determine the cell’s specific membrane capacitance and cytoplasm conductivity.

Figure 4.1 (b) is the impedance amplitude profiles (7 frequencies) measured within 1 second with 8 cells passing through the constriction channel. The excitation voltage of a single frequency is 0.2 V_{pp}, which is intentionally selected to provide a measurable current signal and avoid possible electroporation (electroporation threshold voltage is ~0.5 V [172, 173]). A threshold is defined in this study to be 1.05× basal amplitude (i.e., the amplitude without cell presence in the constriction channel) at 1 kHz (see dash-dotted line in Figure 4.1 (b)). Comparing a signal amplitude value with the threshold, the portions where the
impedance amplitude is higher than the threshold value are considered as cell passage regions. The maximal values of each frequency within the cell passing region are extracted and used to calculate the electrical parameters of the cell.

Figure 4.1 (a) Schematic of the microfluidic system for electrical measurement of single cells. Within the constriction channel, “green” represents the cytoplasm and “red” represents the membrane. “i” is the current through the channel. $R_{cyto}$ represents cytoplasm resistance and $C_{mem}$ represents membrane capacitance; $R_{leak}$ represents the leaking resistance between cell membrane and channel walls; $R_{ch}$ and $C_{ch}$ represent resistance of the medium and parasitic capacitance of the channel. (b) Experimental data showing impedance amplitude profiles (at 7 frequencies) measured within 1 second with 8 cells passing through the constriction channel. Impedance profiles of different frequencies are plotted in different colors.
Figure 4.2 (a) An AML-2 cell is inside the constriction channel. (b) Schematic representation of the simplified geometrical model (“green” and “red” represent cytoplasm and membrane, respectively).

Figure 4.3 (a) The channel without cells passing is modeled as a resistor $R_{ch}$ and a capacitor $C_{ch}$ in parallel. (b) The cell is modeled as a resistor $R_{cyto}$ (cytoplasm) and two capacitors $C_{mem}$ (membrane) in series; $R_{leak}$ represents the leaking resistance between cell membrane and channel walls; $R_{ch}'$ represents the medium’s resistance after a portion of the constriction channel is occupied by a cell. $G$ is a pre-amplifier converting current into voltage signals.
4.3 Materials and methods

AML-2 (acute myeloid leukemia) and HL-60 (human promyelocytic leukemia) cells were chosen for testing in this study. Their comparable sizes permit the evaluation of using their electrical parameters (i.e., specific membrane capacitance and cytoplasm conductivity) for cell classification. Furthermore, the characterization of electrical properties of white blood cells (WBCs) has been reported (e.g., using the electrorotation technique), providing reference data for comparison with our measurements. WBCs behave like a droplet of viscous liquid and are highly deformable [174, 175]; therefore, they can easily pass through the constriction channel (cross-sectional area: 9µm×9µm, the channel is slightly smaller than the cells’ diameter and was lubricated by incubating with 1% BSA to minimize the friction between cell membrane and channels walls). When a WBC is inside the constriction channel, its geometry is well confined by the channel (Figure 4.2 (a)). To quantify the geometry of the tested cells, a simplified geometrical model is adopted [26]. The cell is modeled as a rectangular cube with two quasi-spherical caps fitted smoothly onto the rectangle (Figure 4.2 (b)). The caps of the cell are taken as a quasi-sphere, with an effective radius \( r \) of \( 1/2 \) the side of the constriction channel’s cross-sectional area (4.5 µm in this case). Under this simplification, the effective area of the membrane (red thin shell around the cells contour) that contributes to the cell’s capacitance \( C_{mem} \) includes only the two quasi-spherical caps’ area (determined by the cross-sectional area of the constriction channel); and the elongation \( L \) (see Figure 4.2 (b)) is the only geometrical parameter that varies across different cells, which is only determined by the cells’ volume, assuming Poisson’s ratio is approximately 0.5 [63].
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Figure 4.3 (a) and Figure 4.3 (b) show the equivalent circuit models of the system without and with cells in the constriction area. The electrical models are modified from the equivalent models used in patch-clamp [110] and proves effective for interpreting our experimental setup [176]. The impedance amplitude $|Z|$ of the system at a specific frequency can be calculated as

$$|Z| = \frac{V_{in}}{V_{out}} \times G$$  (1)

where $V_{in}$ is the amplitude of the excitation voltage (0.2 volt in this case), $V_{out}$ is the output of the pre-amplifier, and $G$ (10,000 ohm in this case) is the gain of the pre-amplifier.

When there is no cell present in the constriction region, the channel is equivalent to a resistor $R_{ch}$ and a capacitor $C_{ch}$ connected in parallel. $R_{ch}$ is the resistance of the medium in between the electrodes determined by the conductivity of the medium and geometries of the channel. $C_{ch}$ is the parasitic capacitance of the channel. $R_{ch}$ and $C_{ch}$ can be obtained by fitting the impedance amplitude profiles to Model 1, before aspiration pressure is applied.

As a cell is present inside the constriction channel, it has an elongation length, $L$ and has membrane capacitance, $C_{mem}$ and cytoplasm resistance, $R_{cyto}$. The cell blocks the current within the channel causing the impedance to increase. When a portion of the constriction channel is occupied by a cell, $R_{ch}$ is also changed due to the change of the medium volume. The resistance of the medium in the channel with the presence of a cell ($R_{ch}'$) can be calculated as

$$R_{ch}' = R_{ch} - \frac{1}{\sigma} \times \frac{L}{4r^2}$$  (2)
where $R_{ch}$ is the resistance of the channel without the presence of cells, $R_{ch}'$ is the resistance of the channel occupied by a cell with elongation $L$, $r$ is the radius of the quasi-spherical caps (4.5 μm in this case), $\sigma$ is the conductivity of the medium (1.6 S/m).

At low frequencies, all the capacitive components can be perceived as an open circuit. Thus, the measured low frequency impedance, $R_{low}$, only reflects $R_{leak}$ and $R_{ch}'$ (Eq. 3). $R_{leak}$ can be calculated as

$$R_{leak} = R_{low} - R_{ch}'$$

In Model 2, $C_{ch}$ remains unchanged with or without the presence of cells in the constriction channel. Thus, only the electrical components related to the tested cell remain unknown ($R_{cyto}$ and $C_{mem}$), which can be obtained by fitting the impedance spectroscopy data to Model 2. Under the geometrical assumption, the specific membrane capacitance and cytoplasm conductivity can be estimated according to

$$\text{specific membrane capacitance} = \frac{C_{mem}}{2\pi r^2}$$

$$\text{cytoplasm conductivity} = \frac{L+2r}{4r^2} \times \frac{1}{R_{cyto}}$$

where $R_{cyto}$ and $C_{mem}$ are the resistance and capacitance of the tested cell obtained from curve fitting, $L$ is cell elongation, and $r$ is the radius of the quasi-spherical caps (4.5 μm in this case). The quasi-spherical caps’ surface area is used as the effective area for specific membrane capacitance, and the cross-sectional area of the channel is used as the effective area for cytoplasm conductivity calculation.
Figure 4.4 (a)(b) Impedance amplitude and phase of the constriction channel without and with cells (5 cells of different diameters), measured at 201 frequency points within the range of 1 kHz to 1 MHz. (c)(d) Impedance amplitude and phase measured at 7 selected frequency points (1 kHz, 20 kHz, 50 kHz, 100 kHz, 200 kHz, 300 kHz, 400 kHz), on the same 5 cells as shown in (a) and (b). Experimental data and curve fitting results are plotted in blue and red, respectively.
4.4 Results and discussion

4.4.1 Curve fitting and frequency selection

Cells of various diameters were first ‘parked’ in the constriction channel with careful application of very low aspiration pressure. This allows an impedance spectroscopy (201 frequency points within the range 1 kHz to 1 MHz) of the parked cells to be attained. In the meanwhile, images of the tested cells were taken via microscopy imaging (Olympus IX81, Olympus Canada Inc., Canada), and cell elongation was measured with a custom-designed image processing program. Figure 4.4 shows the impedance amplitude (a) and phase (b) spectroscopy of the constriction channel without and with cells having different diameters. Nonlinear least squares curve fitting (MATLAB, Mathworks, USA) was used to fit the measured impedance to the equivalent circuit models. Before a cell was parked, the impedance profile was measured and fitted to Model 1 (see Figure 4.4 (a)) to determine $R_{ch}$ (0.85 Mohm) and $C_{ch}$ (0.18 pF). When a cell was parked in the channel, the impedance spectroscopy was measured and fitted to Model 2 to determine $C_{mem}$ and $R_{cyto}$. We tested 20 AML-2 cells with 201 frequency points spectroscopy, and the $C_{mem}$ and $R_{cyto}$ were determined to be 1.838±0.240 pF and 0.300±0.057 Mohm, respectively, with all fitting regression coefficients higher than 0.99.

Generally, more frequency points are preferred for impedance spectroscopy to obtain more comprehensive information of the tested system. However, due to the limit of instruments (e.g., HF2IS, Zurich Instrument, Switzerland), measurements can only be made at 7 frequencies. Thus, the 7 frequency points must be properly selected. As shown in Figure 4.4 (a) and Figure 4.4 (b), the system is almost purely resistive at low frequencies (near 1 kHz-10 kHz), wherein the cell membrane acts as an efficient insulator. This can be seen in
the impedance phase of the system (close to 0 degree). In the range of 1 kHz and 10 kHz, the impedance amplitude value does not vary much. Hence, the impedance amplitude at 1 kHz is selected to reflect the resistive characteristic of the system. High frequency (near 1 MHz) results in low impedance of $C_{ch}$, and electrical field lines undesirably obviate the cell in the constriction channel. As a result, the impedance amplitude of the channel with a cell and without a cell becomes rather close, which can barely reflect the cell’s electrical properties. Therefore, more frequency points (20 kHz, 50 kHz, 100 kHz, 200 kHz, 300 kHz, 400 kHz) are selected within the middle frequency range for well capturing the cell’s electrical properties. The impedance amplitude at the selected 7 frequency points of the same 20 cells was fitted to Model 2 (see Figure 4.4 (c) and Figure 4.4 (d)). The calculated $C_{mem}$ and $R_{cyto}$ are $1.836 \pm 0.218 \text{ pF}$ and $0.316 \pm 0.051 \text{ Mohm}$, which are fairly close to the values obtained from 201 frequency points fitting ($1.838 \pm 0.240 \text{ pF}$ and $0.300 \pm 0.057 \text{ Mohm}$). These results prove the validity of the selected 7 frequencies.

4.4.2 Cell elongation measurement using impedance signal

Cell elongation can be measured using high-speed microscopy imaging. However, high-speed cameras (tens of kHz) generate gigabyte data per second and can only record for a few seconds due to limited memory. Processing this massive amount of image data takes tremendous computation efforts and time. Hence, high-speed imaging for measuring cell elongation ($L$) is not a desirable approach. Since the cell membrane acts as an insulate layer at low frequencies (lower than 1 kHz), the impedance increase at 1 kHz is only determined by $R_{\text{leak}}$, which is proportional to cell elongation ($L$). Before high-speed measurements, we correlated the elongation of cells to the impedance increase ($\Delta R$) at 1 kHz by microscopy imaging and manually measuring tens of cells’ elongation in the captured images (see Figure
4.5. The fitted relationship was then used to estimate cell elongation in the subsequent all-electrical high-throughput experiments. Compared with HL-60, AML-2 is less deformable [177], leading to a slightly larger gap between AML-2 cell membrane and the corners of the rectangle-like microchannels. Thus, as shown in Figure 4.5, with the same elongation, AML-2 cells caused a lower impedance increase compared to HL-60 cells, resulting in the different slopes of linear trends between AML-2 and HL-60 cells.

4.4.3 Specific membrane capacitance and cytoplasm conductivity

Our microfluidic system tested AML-2 cells and HL-60 cells. Impedance profiles were recorded and fed into an automated curve fitting program. The program outputs values of specific membrane capacitance, cytoplasm conductivity, cell elongation and norm of the curve fitting. Although intact cells represent the majority in the tested cell suspension, there were also debris, clusters, and apoptotic cells. All the data points shown in Figure 4.5 were measured on intact single cells as verified in experiments via imaging. The data revealed that impedance increase at 1 kHz (ΔR) falls into the range of 0.3 Mohm to 3.5 Mohm for the intact single cell events. Thus, the events with impedance increase at 1 kHz (ΔR) out of this range were regarded as debris or clusters and were excluded from the final data. In the meanwhile, since the $C_{\text{mem}}$ values of the apoptotic cells are so low that the curve fitting program is not able to locate a solution within the reasonable range, the events with exceptionally high curve fitting norm are regarded as apoptotic cells (confirmed via imaging) and excluded from the final results. Using these criteria, approximately 5% of events were excluded. Figure 4.6 (a) shows the scatter plot of specific membrane capacitance vs. cytoplasm conductivity of AML-2 (n=3,249) and HL-60 (n=3,398). Both parameters were quantified and fitted to normal distributions (see Figure 4.6 (b)). The determined specific
membrane capacitance and cytoplasm conductivity of AML-2 and HL-60 are 12.0±1.44 mF/m², 0.62±0.10 S/m and 14.5±1.75 mF/m², 0.76±0.12 S/m, respectively. The difference in specific membrane capacitance values can be attributed to the differences in the two cell types’ surface morphology [178]. Membrane morphology was confirmed to be a primary factor influencing cells’ specific membrane capacitance [81, 82]. Additionally, the different nucleus-to-cytoplasm ratios of these two cell types, as we observed in imaging, could have contributed to the measured differences in cytoplasm conductivity.

4.4.5 Error analysis

Cell elongation is estimated by using the correlation of cell elongation and the impedance increase at 1 kHz (Figure 4.5). The error can be quantified to be

\[
\Delta L = \frac{1}{N} \sum_{i=1}^{N} |y_i - f_i|
\]

where \(\Delta L\) is the average error of cell elongation measurement, \(y_i\) is cell elongation measured via microscopy imaging, \(f_i\) is the predicted value calculated via the fitted relationships, and \(N\) is the total number of data points. According to Eq. 6, the average elongation measurement errors of AML-2 and HL-60 are 2.03 µm and 1.48 µm, respectively. When the cell elongation is used for the estimation of \(R_{ch}'\) (Eq. 2), the error in elongation estimate can cause an error of 0.01-0.03 Mohm of \(R_{ch}'\), which accounts for less than 3.5% of \(R_{ch}'\). Furthermore, \(R_{ch}'\) is much smaller compared with the impedance value of \(R_{cyto}\) and \(C_{mem}\). Thus, the elongation errors’ effect on the electrical component calculation (\(R_{cyto}\) and \(C_{mem}\)) is negligible. However, the elongation error can lead to 7-12% errors in cytoplasm conductivity estimation (Eq. 5).
We further investigated the effect of cell size on the measured specific membrane capacitance and cytoplasm conductivity of AML-2 and HL-60. The diameters of the cells were estimated on the basis of the geometrical model with Poisson’s ratio of 0.5 (Figure 4.6(c)). The cells within the diameter range of 10.5µm to 13µm were chosen, since the majority of these two types of cells fell in this range (>70% for AML-2 and >80 for HL-60), and within this range, the cell diameters of these two cell types heavily overlapped. The range of interests was divided into five sub-ranges (10.5-11µm, 11-11.5 µm, 11.5-12 µm, 12-12.5 µm, 12.5-13 µm. For a certain cell type, the specific membrane capacitance and cytoplasm conductivity values of the five diameter sub-ranges are all fairly close to the population’s average, and the differences of the specific membrane capacitance and cytoplasm conductivity values for AML-2 and HL-60 are almost the same across all the sub-ranges, indicating the different electrical properties of AML-2 and HL-60 measured using our device truly result from the different electrical properties of the cells, rather than the diameter variation.

Specific membrane capacitance and cytoplasm conductivity values are inherent electrical properties of the cells, which should ideally be completely independent of cell size. However, as the cell diameter increases, our measurement results show a very minute increase trend of the specific membrane capacitance and cytoplasm conductivity values for both AML-2 and HL-60 cells. This trend may result from measurement errors caused by the use of the simplified geometrical model. The quasi-spherical cap’s surface area is used as the effective area for all the cells to calculate the specific membrane capacitance. Cells with larger diameters may result in slightly larger effective area (vs. cells with smaller diameters) due to the squeeze of the channel, which can cause increase in specific capacitance membrane
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calculation (Eq. 4). The cytoplasm conductivity estimation assumes a uniform current distribution inside the cell membrane. The different quasi-spherical caps may alter the current distributions inside the cytoplasm, and consequently affect the calculated cytoplasm conductivity value. Overall, the measurement errors caused by cell diameter variation on the determined specific membrane capacitance and cytoplasm conductivity values are small. Thus, the electrical property values are valid for representing the population of a certain cell type. In fact, the results obtained using our technique are consistent with the values reported in electrorotation studies, which are also based on the single shell model (specific membrane capacitance of HL-60: 15 ± 1.9 mF/m², 15.6 ± 0.9 mF/m²; specific membrane capacitance of human granulocytes: 11.0 ± 3.2 mF/m²; and cytoplasm conductivity of human granulocytes: 0.60 ± 0.13S/m [81, 179]).

4.4.6 Cell type classification

Since AML-2 (diameter: 12.5±1.3 µm) and HL-60 (diameter: 11.4±0.93 µm) cells have comparable diameters, the Coulter counter technique produced a low classification success rate of 67.7%. With additional information on cells’ electrical properties, these two cell types were better classified. A back propagation neural network was used for pattern recognition (MATLAB, MathWork, USA). The input data have three parameters (diameter, specific membrane capacitance, and cytoplasm conductivity) measured on each cell. Cell type classification success rates were 67.7% (diameter only), 84.4% (diameter + cytoplasm conductivity), 88.6% (diameter + specific membrane capacitance), and 93.0% (diameter + cytoplasm conductivity + specific membrane capacitance), suggesting that using specific membrane capacitance and cytoplasm conductivity can significantly improve the classification success rate (vs. only using cell diameter/size as in Coulter counter). This result
is not surprising, for the two cell types within the same diameter range, their inherent electrical properties (specific membrane capacitance: P value $<10^{-150}$, cytoplasm conductivity: P value $<10^{-70}$) are significantly different. These inherent electrical properties contain additional information (cell membrane and cytoplasm properties) to cell size.

4.5 Conclusion

This chapter demonstrated a new technology for rapid characterization of single-cell electrical properties (specific membrane capacitance and cytoplasm conductivity). The cells are aspirated through a constriction channel, and impedance profiles at 7 different frequencies are measured simultaneously. Geometrical and electrical models were developed to quantify the specific membrane capacitance and cytoplasm conductivity from the experimental impedance data. Compared with previously reported techniques for single-cell electrical characterization, the speed of our system is significantly higher (5-10 cells per second vs. minutes per cell). A total of 3,249 AML-2 cells and 3,398 HL-60 cells were tested, and the specific membrane capacitance values were determined to be 12.0±1.44 mF/m$^2$ and 14.5±1.75 mF/m$^2$, respectively, while cytoplasm conductivity values were determined to be 0.62±0.10 S/m and 0.76±0.12 S/m. The measurements can be used for cell type classification or provide additional information on cells’ physiological conditions.
Figure 4.5 Experimental correlation between cell elongation and impedance increase at 1 kHz of AML-2 (blue) and HL-60 (red).
Figure 4.6 (a) Scatter plot of specific membrane capacitance vs. cytoplasm conductivity of AML-2 and HL-60. Color coding represents data distribution densities. (b) Histograms of specific membrane capacitance and cytoplasm conductivity of AML-2 (red) and HL-60 (cyan) fitted with normal distributions. (c) Histograms of AML-2 (red) and HL-60 (cyan) cell diameters fitted with normal distributions.
Chapter 5


5.1 Introduction

In transfusion medicine, red blood cells (RBCs) collected from blood donors are stored and preserved in a blood bank. Every year in the U.S. and Canada, over 14 million units of RBCs are administered to more than 5 million patients [180]. Present regulations in Canada and the U.S. specify 42 days as the shelf life for stored blood [180, 181]. However, it has been suggested that during storage red blood cells undergo morphological, structural, and functional changes, which may induce clinical complications and adversely affect patient mortality [182-185]. For instance, transfusion of RBCs stored for more than two weeks was found associated with a significantly increased risk of postoperative complications as well as reduced short-term and long-term survival of cardiac surgery patients [186].

The degradation of stored RBCs is known as the storage lesion [183]. Although the clinical consequences of storage lesions remain controversial [182], intensive research has shown how parameters, which govern RBCs’ metabolic ability and oxygen delivery capacity,
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such as 2,3-DPG, potassium, pH, HbO₂ saturation, RBC ATP, RBC NO, SNO-Hb, and haemolysis, change over the life span of stored RBCs [146, 180, 187, 188]. In addition to these biochemical properties, the biconcave shape and high deformability of RBCs are also crucial for their physiological activities and functionality.

The study on the deformability of stored RBCs dates back to the 1960s. La Celle and Weed investigated the progressive alteration of stored RBC deformability by using a micropipette [189, 190]. The hydraulic pressure required to aspirate the RBCs through the micropipette was used as a deformability indicator. Their results show that the RBCs that remained biconcave disc-shaped during storage have a deformability similar to that of fresh RBCs; however, those RBCs that became more spherical had decreased deformability. Optical tweezers were also applied to characterize the deformability of 0 days and 35 days stored RBCs by optically stretching the cells, wherein higher membrane elasticity and viscosity were observed in 35-day old RBCs [137]. More recently, ektacytometry was used to study the deformability of stored RBCs. Ektacytometry consists of two rotating plates with a small gap in-between. RBCs adhere to the bottom of the gap, and shear stress elongates the RBCs. The extent of cell elongation is measured as an indicator of RBC deformability. Using ektacytometry measurements, the progressive elongation change of stored RBCs over 42 days storage period was reported, demonstrating that RBCs become harder to elongate when stored longer [180, 191-193].

Measuring the deformability of stored RBCs using micropipette and optical tweezers is tedious and skill-dependent. More importantly, the slow measurement speed (minutes to tens of minutes for testing one cell) makes these techniques infeasible to obtain sufficient information of the highly heterogeneous blood cell population [13, 17, 194]. As stored RBCs
age, the cells change their morphology progressively from biconcave to more spherical (spheroechinocytes) [184, 188, 195]. Even for the cells within the same stored blood sample, their morphology change varies significantly. Hence, testing only a few cells from a blood sample cannot objectively reveal deformability changes of the sample.

Compared to micropipette and optical tweezers, ektacytometers are relatively easy-to-use. However, ektacytometry measurement is limited to approximately 50-60 RBCs per test. In ektacytometry, elongation index measured via laser diffraction is the only parameter for indicating RBC deformability [164, 196]. The definition of elongation index becomes improper when RBCs’ shapes become less regular [197-199]. Furthermore, ektacytometry requires RBCs to settle and adhere to the bottom of the ektacytometer chamber in order to elongate the cells under shear stress. This deformation mode is not physiologically relevant since in vivo RBCs are folded when flowing through human microcapillaries with diameters comparable to or smaller than RBCs [141, 155, 157]. It is known that the mechanical properties of RBCs can differ significantly when deformed under different modes (e.g., extension or folding) [200, 201].

This chapter describes the folding of stored RBCs on a microfluidic device. Fresh and stored RBCs were pressure-driven to flow through microfluidic channels (cross-sectional area: 8 µm×8 µm). Hydrodynamic focusing within the microchannel controls the orientation and position of individual RBCs. High-speed imaging (5,000 frames/sec) captures the dynamic deformation behavior of the cells, and together with automated image analysis enables the characterization of over 1,000 RBCs within 3 minutes. Multiple parameters including deformation index (DI), time constant (recovery rate after an RBC exits the channel), and circularity of the individual cells were quantified. Compared to existing stored
RBC deformability studies, our results include a significantly higher number of cells (>1,000 cells/sample vs. a few to tens of cells/sample) and, for the first time, reveal deformation changes of stored RBCs when traveling through human-capillary-like microchannels. The correlation between deformability and morphology of stored RBCs is also reported.

5.2 System overview

Figure 5.1 (a) shows a schematic of the microfluidic device for studying RBC deformability changes during storage. The central channel is 160 µm long and has a cross-sectional area of 8 µm×8 µm. The recovery region has a cross-sectional area of 200 µm×8 µm, where the deformed RBCs gradually recover to their original shape. Two focusing channels are integrated to center and orient the RBCs. The loading and focusing channels are connected to a custom-developed precision pressure source using water tanks. When RBCs flow through the central channel, RBC images are captured by a high-speed camera (5,000 frames/sec) through an inverted microscope. Figure 5.1 (b) shows the dynamic process of RBC deformation. When the cell entered the channel, its position was close to one of the microchannel walls. After the focusing unit, the cell was centered and adjusted to the ‘standing’ orientation. It then underwent symmetrical shear stress, resulting in a parachute-like shape near the exit. When the cell exited the microchannel, shear stress was released, and the RBC gradually recovered to its original shape.

In order to quantify the deformation behavior of RBCs, deformation index (DI) is defined as DI=L/D [see Figure 5.1 (a)] in the last frame of image right before the cell exits the microchannel and enters the recovery region. Recovery time constant (tc) is determined by exponentially fitting DI values with respect to time. Circularity, defined as circularity=4π×Area/Perimeter² (circularity=1 means a perfect circle), is measured
experimentally in the last frame of image right before the cell exits the region of interest to depict the morphology of each stored RBC.

Figure 5.1 (a) Schematic of the microfluidic device for studying deformability changes of stored RBCs. Hydrodynamic focusing centers the cell and adjusts it to the ‘standing’ orientation. The RBC is ‘folded’ into a parachute-like shape when pressure-driven through the 8 µm×8 µm central channel. Deformation index (DI=L/D) is defined to measure RBC deformation (top-left). Time constant (tc) of each individual cell is determined by fitting DI value changes during the cell shape recovery process to an exponential function, after the cell exits the microchannel. (b) Experimental images showing the centering, orienting, folding, and shape recovering of an RBC.
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5.3 Methods and materials

5.3.1 Blood samples

Stored blood samples were collected using techniques consistent with the Technical Manual of the AABB [202]. Briefly, venous blood samples (500 ml ±10%) were collected from healthy donors in CP2D anticoagulant. After separation of plasma and buffy coat, the RBCs were suspended in saline-adenine-glucosemannitol (SAGM) (110 ml), and then stored in a blood bank refrigerator at 4°C. For each test, an RBC aliquot was removed and tested at desired time intervals [180, 188]. After an RBC sample was drawn from the aliquot, it was diluted with PBS with 1% w/v BSA (RBC sample:PBS=1:100) and incubated for 10 min at room temperature to prevent adhesion to channel walls. Fresh blood samples were obtained from healthy donors (Mount Sinai Hospital, Toronto, Canada) and tested following the same procedure with the stored RBC samples. All samples were tested within one hour after incubation.

5.3.2 Experimental methods

The microfluidic device was constructed with PDMS using standard soft lithography, as described elsewhere [203]. The device consists of two layers of different thickness. The thickness of the testing areas is 8 µm, while the thickness of the loading channels is 50 µm for reducing flow resistance. After the RBCs were loaded into the device inlet, a custom-developed water tank applied pressure (2.5 kPa) to drive the cells through the microchannels. Cell images were captured at a speed of 5,000 frames/sec (416 pixel by 142 pixel) via a high-speed camera (HiSpec 1, Fastec Imaging Corp., U.S.), under a 60× objective of an inverted microscope.
A custom-designed MATLAB image processing program was developed for automated RBC image analysis. For each video, the region of interest (ROI) is defined as the microchannel region excluding the PDMS area. After Gaussian filtering, canny edge detection with adaptive thresholding is applied to extract cell edges. All the edge points inside the ROI are accumulated along the X and Y axes, respectively. The peak regions in the X and Y curves indicate the coarse cell region, based on which the fine cell region is obtained by iterations of localizing the active contours of the cell. Length, width, area, and perimeter are measured for each RBC to quantify its DI, time constant (tc), and circularity.

Figure 5.2 Efficiency of hydrodynamic focusing. Histogram of central distance (i.e., the distance between the center of the cell and microchannel’s central line) of RBCs before (blue) and after (red) the focusing unit (n=1,500). Central distance of zero means the cell is perfectly centered within the microchannel. The percentage of RBCs near the microchannel center was increased significantly by hydrodynamic focusing.
5.3 Results and discussion

5.3.1 Hydrodynamic focusing efficiency

The shape of RBCs flowing through small capillaries is a function of flow rate, initial position relative to the central line of the capillary, and the diameter of the capillary [141, 151, 204, 205]. For consistency, RBCs need to be centered in the microchannel and deformed symmetrically. If cells are asymmetrically deformed, the definition of DI=\(L/D\) becomes less appropriate for describing their deformation behavior [141]. Experiments demonstrate that RBCs close to the central line of the microchannel reveal a more symmetrically deformed shape while cells near channel walls usually are asymmetrically deformed. Cells near the channel walls at the entrance typically stay near the channel wall until exiting the channel.

When RBCs enter the channel, their positions are random. Hence, hydrodynamic focusing near the inlet (see Figure 5.1) was used to better position RBCs close to the central line of the microchannel. Figure 5.2 shows a histogram of central distance (i.e., the distance between the cell center and the microchannel’s central line) of RBCs before (blue) and after (red) the hydrodynamic focusing unit (\(n=1,500\)). The percentage of RBCs near the microchannel center was increased significantly by hydrodynamic focusing, and the number of cells more than two pixels away from the channel center was significantly reduced. The data presented in this chapter only include those RBCs that were no more than two pixels away from the central line of the microchannel.

5.3.2 Time constant

After the RBC enters the recovery region, the time constant (\(tc\)) of each RBC is determined by fitting its DI values over time to an exponential function, in order to characterize the
recovery rate of the cell. The exponential model is adapted from the standard Kelvin–Voigt model for describing the recovery properties of RBCs [206, 207]. Figure 5.3 shows two sets of data, one from a biconcave RBC (blue) and the other from a spherical RBC (red) due to storage. The first data point on each curve was obtained from the image frame right after the cell exits the microchannel. For the RBCs shown in Figure 5.3, time constant (tc) of the spherical RBC is 0.267 ms, while the tc value of the biconcave RBC is 0.625 ms.

It is worth noting that the time constant of RBCs reported in the literature was approximately 100 ms [208-210]. In contrast, the time constant we quantified ranges from 0.1 ms to 1 ms. In previous studies, DI was typically measured starting from 100 ms after an RBC was released from deformation until the shape completely recovered (several seconds) [208, 209]. In our experiments, enabled by high-speed imaging, the rapid recovery process immediately after an RBC is released was captured (from 0.2 ms to 10ms). During this period, RBCs recover much faster than in the later period (e.g., after 100 ms as in earlier studies). As a result, the captured fast dynamics of RBC deformation more authentically revealed the cell shape recovery behavior, and exponential fitting resulted in time constant values orders of magnitude lower than those from earlier results in the literature.
Figure 5.3 Shape recovery of a biconcave RBC (blue) and spherical RBC (red) fitted to an exponential model. The spherical shape of the cell was due to morphological change during blood storage. Time constant (tc) of the spherical RBC is 0.267 ms, and the tc value of the biconcave RBC is 0.625 ms. The relationship between deformability change and morphology change is discussed in the next section.
Figure 5.4 (a)(b) Scatter plots of time constant vs. circularity; deformation index (DI) vs. circularity, for the same blood sample stored for 1 week (n=1,038), 3 weeks (n=1,027), and 6 weeks (n=1,001). (c) Distribution of time constant, circularity, and DI.
5.3.3 Changes in RBC deformation index, time constant, and circularity over blood storage

Figure 5.4 summarizes results from testing the same blood sample stored for 1 week (n=1,038), 3 weeks (n=1,027), and 6 weeks (n=1,001). The scatter plots show tc vs. circularity [Figure 5.4 (a)] and DI vs. circularity [Figure 5.4 (b)]. Within each sample, both tc and DI show a decreasing trend as circularity increases, indicating that morphological changes of RBCs are a factor that causes RBC deformability changes over blood storage. Figure 5.4 (c) shows the distribution of these three parameters. It can be seen that the average time constant becomes lower as the RBCs are stored longer. The lower time constant may be attributed to ATP loss. It has been proven that the depletion of ATP alters its binding to spectrin–actin, which modifies RBC’s cytoskeleton structure [211-213], resulting in RBC stiffening and faster recovery [208, 214]. During storage, RBCs undergo a number of biochemical changes, including ATP depletion [215, 216], which may be a cause of the lower time constants of older RBCs.

The average circularity of fresher and older RBCs is not significantly different; however, the distribution (i.e., standard deviation) becomes wider. Thus, the distribution width of circularity (circularity-DW) can possibly be used as an indicator of stored RBC quality or age. The alteration of circularity distribution over time is mainly contributed by RBC morphology changes. Based on experimental observation, as stored RBCs age, they first swell and then progressively change to a sphere-like shape. A portion of the aged RBCs became more spherical, making their circularity approach one. There were also RBCs appearing isotropically enlarged from storage, and these RBCs typically revealed a higher DI value.
When they pass through the microchannel, their deformed shape resulted in low circularity. The more spherical cells and isotropically swelling cells increased circularity-DW.

No significant difference in DI was found (DI: 1.227 for 1-week, 1.221 for 3-week, and 1.219 for 6-week) [Figure 5.4 (c)]. This implies that when stored RBCs are transfused into patients, they are able to flow through microcapillaries with a similar folding capability. However, their stretching capability might become poorer, according to the lower elongation index (EI) measured with ektacytometry [180, 191-193]. The insignificant folding DI change of older RBCs (vs. fresher RBCs) as quantified in our work, and the significant stretching EI change of older RBCs (vs. fresher RBCs) as reported in ektacytometry measurements can be due to the fundamentally different cell deformation modes [200, 201]. Although the depletion of ATP alters RBC cytoskeleton, the stretching EI change might not be a concern in transfusion medicine since the stretching mode is not in vivo like and can be physiologically irrelevant.

We further investigated the effect of RBC morphology change (circularity) on time constant. Figure 5.5 shows the average and standard deviation of measured tc values within each circularity range (divided into five sub-ranges). Both fresher and older RBCs with higher circularity reveal lower time constant. Due to intrinsic property changes, within each circularity range, older RBCs show consistently lower time constants, compared to fresher RBCs.

Finally, five blood samples were tested from fresh to 8 weeks’ storage, at time intervals of every two weeks (n>1,000 per time interval per sample). As shown in Figure 5.6, time constant decreases and circularity-DW (distribution width) increases over blood storage. Significant differences exist between neighboring data points (p<0.05), demonstrating that
time constant and circularity-DW can possible be used as indicators of RBC storage age or stored RBC quality. Standard deviations shown in Figure 5.6 can be attributed to blood donor variations and variations in blood processing procedures [217, 218].

Figure 5.5 Comparison of time constants for the same blood sample stored for 1 week (n=1,038), 3 weeks (n=1,027), and 6 weeks (n=1,001). Circularity is divided into five sub-ranges. Error bars represent standard deviation. In the last circularity range (>0.8), the group of 1 week old RBCs is not present because no RBC has a circularity higher than 0.8 in 1 week old sample.
Figure 5.6 Time constant (a) and circularity-DW (b) alteration over time. Each data point was obtained from 5 blood samples and over 1,000 cells were tested within each sample. Significant differences exist between neighbor data points (p<0.05). A relatively larger change between fresh and 1-2 week samples was found, compared to the steady changes over time during storage.
5.4 Conclusion

The deformability changes of stored red blood cells (RBCs) were studied using a human-capillary-like microfluidic channel. High-speed imaging system and automated image processing were used to quantify multiple parameters, enabling a higher measurement speed. Fresh and stored blood samples (up to 8 weeks) were tested. Besides large sample sizes, our study, for the first time, revealed deformation behavior changes of stored RBCs when traveling through human-capillary-like microchannels. Although existing literature consistently reported stretching deformability change of stored RBCs, our results show that no significant difference exists in their folding deformability. Furthermore, we report that significant changes in time constant (i.e., recovery rate) and circularity distribution width (i.e., heterogeneity of morphology) can be useful parameters for quantifying stored RBC quality or age.
Chapter 6

6. Decreased deformability of lymphocytes in chronic lymphocytic leukemia

6.1 Introduction

Over the past decade, there is growing evidence showing that cells undergo biophysical property changes during disease progression [219, 220]. For example, the increased deformability of malignant cancer cells, as compared to benign cells, facilitates their migration and invasion [194, 221]; and in sickle cell disease, sickled red blood cells due to their higher stiffness can cause vaso-occlusion [222]. Biophysical property changes of various leucocytes under a number of pathological conditions have been reported. Neutrophils were found to be significantly stiffened by formylmethionyl-leucyl-phenylalanine (FMLP) activation, and thereby took longer time to pass 5 μm capillary pores [223]. Neutrophils’ deformability can be severely impaired in sepsis patients, and thus migration capability was consequently jeopardized [224, 225]. Research has also shown that immature granulocytes and lymphocytes’ inability to traverse micropipettes due to compromised deformability, which can cause leukocclusive events, abnormal intravascular leukocyte aggregation and clumping in vasculature [226]. A recent micropipette aspiration
study of lymphocytes from diabetic mice revealed that diabetic lymphocytes are stiffer than control cells, which is associated with several clinical complications [227].

Leucocytes are nonadherent cells and can be tested with atomic force microscopy (AFM) when mechanically immobilized in microfabricated wells [228, 229]. Using this technology, the stiffness alteration of acute lymphoblastic leukemia (ALL) cell lines (HL-60 and Jurkat) caused by chemotherapy was quantified [177]. The study concluded that after exposure to chemotherapy, leukemia cells possess increased stiffness, and the type of chemotherapy can affect stiffness kinetics. The increased cell stiffness in ALL patients with leucostasis symptoms was also shown by using the AFM technique [12]. Most recently, data obtained using optical tweezers provided preliminary evidence that leukemic hematopoietic cell populations in normal and leukemia patients with distinct primitiveness exhibited differential deformability [230].

To understand the pathophysiology involving biophysical properties of cancerous leukocytes (leukemia), existing studies either performed measurement on cell lines instead of patient samples or tested a very limited number of cells. This chapter presents the first study of stiffness/deformability changes of lymphocytes in chronic lymphocytic leukemia (CLL) patients based on the measurement of more than 1,000 cells per patient sample. The results reveal that lymphocytes from CLL patients have a higher stiffness (i.e., lower deformability), as compared to their counterparts in healthy samples, which is different from the known knowledge on other types of metastatic cells (e.g., breast and lung cancer cells) whose stiffness becomes lower as metastasis progresses.
6.2 Materials and methods

Peripheral blood samples were collected from patients diagnosed with CLL following the protocol of standard blood tests (anticoagulant with 1.5 mg/ml EDTA (ethylenediaminetetraacetic acid)). Mononuclear cells were isolated using a standard density-gradient protocol (Ficoll-Paque PLUS, GE Healthcare Bio-science, AB). Briefly, 4 ml of diluted whole blood (2 ml blood : 2 ml PBS) was carefully layered on 3 ml Ficoll-Paque PLUS, and then centrifuged at 400 g for 30 mins, which leaves the lymphocyte layer at the interface. The lymphocyte layer was re-suspended in 6ml PBS and centrifuged at 80 g for 10 min to remove platelets. After removing the supernatant, the cell pellet (<5% monocytes and erythrocytes, >95% lymphocytes) was re-suspended in PBS with 1% w/v BSA (with a final density of 3 M/ml) and kept at room temperature for 30 min to reduce adhesion to channel walls.

Microfluidic devices were fabricated via standard PDMS soft lithography. The two stages of the microchannel [Figure 6.1(a)] have cross-sectional areas of 5 µm×5 µm and 8.5 µm×8.5 µm, respectively [205]. Two Ag/AgCl electrodes were used to measure resistance changes. Due to the blockage of electrical current [203], a single cell generates a pair of resistance peaks [Figure 6.1(b)(c)] when driven through the channel under a negative pressure (3 kPa). The small peak and the large peak in the resistance profile correspond to the resistance increase when a cell passes through the 8.5 µm×8.5 µm channel and the 5 µm×5 µm channel, respectively. The height of the smaller peak (∆R) was used to measure cell volume according to the Coulter counter principle; and the width of the larger peak was used to determine transit time, ∆t.
Figure 6.1(b) shows experimental data measured within 1 sec (11 cells). Figure 6.1(c) is the zoomed-in view of the circled area in Figure 6.1(b). To extract the actual cell volume, finite element modeling was conducted using COMSOL. In finite element modeling, dielectric spheres with known sizes (diameter ranging from 5.0 µm to 8.5um) were simulated and corresponding electrical resistance values were determined. Cells with a diameter larger than 8.5 µm or smaller than 5 um were monocytes and red blood cells, and hence excluded in data processing.

Figure 6.1 Microfluidic system for electrically measuring lymphocytes volume and transit time. When a lymphocyte is driven through the measurement units under a negative pressure (3 kPa) (also see SI Video 1), the total electrical resistance of the channel increases due to the blockage of electrical current. (b) Experimental resistance data recorded within 1 second. Zoom-in of the circled area is shown in (c). Cell volume and transit time are determined by measuring ∆R and ∆t.
CHAPTER 6, LYMPHOCYTES IN CHRONIC LYMPHOCYTIC LEUKEMIA

6.3 Results and discussion

Figure 6.2(a) shows scatter plots of transit time vs. cell volume for lymphocytes from a control/healthy sample (n=1,048) and a CLL sample (n=796). The distributions of cell volume and transit time are presented in Figure 6.2(b). Transit time is mainly affected by cell volume and cell deformability. We first investigated whether cell volume alone can explain the transit time variation. Within the same sample, lymphocytes with larger volume indeed took longer time to travel through the microfluidic channel. Our measured transit time exhibited a power law dependence on cell volume in agreement with previous studies where cell is modeled as a viscous liquid drop \[231, 232\]. It is worth noting that within the same sample, a 2 µm change in cell diameter caused transit time to span more than an order of magnitude, which indicates the dominant effect of cell size/volume on transit time. However, with the same diameter, transit time of the lymphocytes varies by 3-4 folds, which indicates the effect of cell deformability differences. As shown in Figure 6.2(a), cell diameter and transit time of the control sample were measured to be 7.20±0.56 µm and 4.88±1.35 ms; whereas the volume and transit time are 6.96±0.62 µm and 5.41 ±1.50ms for the CLL sample. Cells in the CLL sample are smaller compared to cells in the control sample but take longer time to pass through the 5 µm × 5 µm channel. This fact led us to hypothesize that lymphocytes in CLL patients have decreased deformability.
Figure 6.2 (a) Scatter plots of transit time vs. cell diameter for lymphocytes from a control/healthy sample (n=1,048) and a CLL sample (n=796). Transit time exhibits a power law dependence on cell volume/diameter. (b) Histograms of cell diameter and transit time of the control and CLL samples. Both volume and transit time follow normal distributions.
Figure 6.3 Transit time (a) and cell volume (b) measured from 5 control/healthy samples (red) and 5 CLL samples (cyan). For each sample, the three lines of the box represent 75 percentile, median, and 25 percentile; the whiskers represent the locations of maximum and minimum (n≈1,000 for each sample). The average median values of transit time (c) and cell diameter (d) of the 5 control samples and the 5 CLL samples are 3.50 ± 0.36 ms vs. 5.13 ± 0.98 ms and 7.36 ± 0.07 µm vs. 7.15 ± 0.13 µm, respectively (*p < 0.05, error bars represent standard deviation of the mean value). In general, cells in CLL samples are slightly smaller than cells in control samples but reveal a longer transit time, indicating that they are less deformable.
Cell volume and transit time from 5 control samples and 5 CLL samples were measured using the microfluidic system. As shown in Figure 6.3(a)(b), for each sample, the three lines of the box represent 75 percentile, median, and 25 percentile. The whiskers represent the locations of maximum and minimum. Figure 6.3(c)(d) summarize average group median values of transit time and cell volume with error bars representing standard deviation of the mean. Compared to control samples, CLL samples have a smaller cell volume (7.15 ± 0.13 µm vs. 7.36 ± 0.07 µm) but a higher median transit time (5.13 ± 0.98 ms vs. 3.50 ± 0.36 ms). The volume measurement results fall in the same range as reported in previous studies\cite{233}. Statistical difference exists between two populations (*p < 0.05).

Furthermore, as shown in Figure 6.3(a), 75 percentile, median, and 25 percentile cell transit time values were all higher for CLL samples, demonstrating that the measured transit time difference reflects entire cell populations tested in this work. Cell transit time variance is more prominent in CLL samples, which is likely attributed to the increased heterogeneity caused by accumulated mutations during malignancies \cite{234}. Note that since none of the CLL patients showed leukostasis symptoms, all the lymphocytes were able to pass through the microchannel \cite{30}.

Surface properties can influence friction between cell membrane and microfluidic channel walls, and hence possibly contribute to transit time differences. It has been reported that changing the coating of channel surfaces from PEG to PLL can cause cell entry velocity and transit velocity to decrease \cite{231}. In our work, the isolated lymphocytes were incubated in PBS with 1% BSA for 30 min to reduce friction \cite{205}. Before loading cells, the microfluidic channel was also perfused with PBS+1% BSA for 30 min to further reduce friction force of control and CLL lymphocytes. To confirm the measured transit time
difference (control vs. CLL) truly reflects cell deformability/stiffness, lymphocytes from a control sample and a CLL sample were tested using AFM indentation.

Lymphocytes’ stiffness was measured when the cells were immersed in PBS at room temperature using an AFM (Bioscope Catalyst, Santa Barbara, CA) mounted on an inverted microscope. The cells were mechanically immobilized within microwells [228, 229]. AFM indentations were conducted using silicon nitride cantilevers with a nominal spring constant of 0.03N/m. The elastic moduli of the lymphocytes were quantitatively determined by fitting force-indentation curves using the Hertz model for a pyramidal tip. Figure 4(a) shows experimental indentation curves of two representative lymphocytes from the control sample and the CLL sample. The elastic moduli of control and CLL samples are 2.92 ± 0.38 kPa and 5.37 ± 0.38 kPa, respectively, as summarized in Figure 6.4(b). This difference in elastic modulus confirms that lymphocytes in CLL patients have a higher stiffness than lymphocytes in control samples, and the higher stiffness contributes to the longer transit time of CLL lymphocytes.

Difference in actin filament density was often thought responsible for deformability differences between two cell types [231, 235]. However, a decrease in actin content of lymphocytes from CLL patients was previously found [236]. In addition, no connection was found between actin level and cell deformability in human leukemia cell lines [228]. Recently, vimentin (intermediate filaments) was shown to have dominant influence on mouse lymphocyte deformation [237]. In our study, we noticed that the nuclei of CLL lymphocytes occupy almost the entire cell (Figure 6.5). As a result, when they flow through the microfluidic channel, the nuclei of CLL lymphocytes could have contributed more significantly to transit time since cell nuclei are known to be significantly stiffer compared to
cytoplasm and cell membrane [226, 238, 239]. Thus, the enlarged nuclei may be a factor that contributes to the longer transit time observed for CLL lymphocytes. However, the exact pathological causes and the underlying mechanism of the observed deformability difference in lymphocytes from healthy and CLL patients necessitate further investigation.

Figure 6.4 (a) Experimental AFM indentation curves of two representative lymphocytes, one from control sample and one from CLL sample. The elastic moduli of lymphocytes were quantitatively determined by fitting force-indentation curves to standard Hertz model for a pyramidal tip. (b) Elastic modulus values of the control sample (n = 10) and the CLL sample (n = 14) are 2.92 ± 0.38 kPa and 5.37 ± 0.38 kPa, respectively (*p < 0.05, error bars represent standard deviation of the mean value).

Figure 6.5 Lymphocytes from control (a) and CLL (b) samples stained with Wright/Giemsa Stain.
6.4 Conclusion

This chapter reports stiffness/deformability differences of lymphocytes from healthy donors and chronic lymphocytic leukemia (CLL) patients. Microfluidic measurement was used to quantify cell volume and transit time of thousands of lymphocytes from control and CLL samples. The results reveal that CLL lymphocytes have higher stiffness (i.e., lower deformability), as compared to lymphocytes in healthy samples. Their higher stiffness was also confirmed via AFM indentation. This study demonstrates that at the single cell level, leukemic metastasis progresses are accompanied by biophysical property alterations.
Chapter 7

7. Conclusions

7.1 Contributions

1. Developed microfluidic systems for high-throughput biophysical (mechanical and electrical) characterization of single cells (i.e., red blood cells, leukocytes). Compared to existing techniques, the proposed systems improves the testing throughput by about two orders of magnitude and are capable of simultaneous characterization of mechanical and electrical parameters of single cells. It also proves that quantification of multiple biophysical parameters significantly improves the cell type classification success rate.

2. Designed a microfluidic system for electrically measuring the deformability of red blood cells (RBCs). The experimental results proved the capability of the system for distinguishing different RBC populations based on their deformability with a throughput of ~10 cells per second.

3. Demonstrated a technique for single-cell electrical property (specific membrane capacitance and cytoplasm conductivity) characterization at a speed of 5–10 cells/s.
The results also demonstrate that the quantification of specific membrane capacitance and cytoplasm conductivity can enhance cell.

4. Quantified the deformability changes of red blood cells during blood storage in human-capillary-like environment. The results demonstrate that the deformation index of RBCs under folding does not change significantly over blood storage. However, significant differences exist in time constants and circularity distribution widths.

5. Studied stiffness/deformability differences of lymphocytes from healthy donors and chronic lymphocytic leukemia (CLL) patients. Microfluidic measurement was used to quantify cell volume and transit time of thousands of lymphocytes from control and CLL samples. The results reveal that CLL lymphocytes have higher stiffness (i.e., lower deformability), as compared to lymphocytes in healthy samples.

7.2 Future directions

1. To further improve the throughput and sensitivity of the microfluidic systems for red blood cells (RBCs) deformability measurements. To model the dynamic process of the red blood cells passing through microchannels and extract material properties (such as shear modulus and the viscosity) of the RBCs from experimental data.

2. To conduct biophysical measurement of diseased RBC samples (such as malaria and sickle cells). To reveal the biophysical property differences between diseased samples and healthy samples. And to explore the feasibility of utilizing biophysical properties for diagnostic application.

3. To design portable, easy-to-use and low-cost microfluidic system for single-cell electrical properties measurement. To implement the system for various clinical
CHAPTER 7, CONCLUSIONS

applications (such as complete blood counting, leukemia cells classifications). To improve the system by adding cell volume measurement unit.

4. To systematically conduct deformability study of the stored red blood cells samples. To reveal the intrinsic association between the biochemical properties (such as ATP, NO and 2,3 DPG) and deformability alteration. To study the effect of donor variations (such as ages and gender) on the stored RBCs’ quality.

5. To reveal the underlying mechanisms of the altered biophysical properties of the lymphocytes in chronic lymphocytic leukemia patients. To further study the association between the clinical complications and the lower deformability of the leukemic lymphocytes.
8. Appendix

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